#15

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Philip Richard ABRAHAM, et al.

Appln. No.: 09/493,211 Group Art Unit: 1653

Confirmation No.: Unassigned Examiner: C. Kam

Filed: January 28, 2000

For: NOVEL SYNTHETIC PEPTIDES WITH ANTIMICROBIAL AND ENDOTOXIN NEUTRALIZING PROPERTIES FOR MANAGEMENT OF THE SEPSIS SYNDROME

SUBMISSION OF EXECUTED DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith is an executed Declaration Under 37 C.F.R. §1.132 signed by Philip Richard ABRAHAM.

Respectfully submitted,

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Date: January 16, 2002

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DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Philip Richard ABRAHAM, hereby declare and state:

THAT I am a citizen of The Netherlands;

THAT I have received the degree of Ph.D. in 1993, from Vrije Universiteit, Amsterdam;

THAT I have been employed by Academic Medical Center, University of Amsterdam, since March 1, 1995, where I hold a position as Senior Scientist, with responsibility for Anti-infective Peptide Development.

I am familiar with claims 1-39 pending in the present application.

I am familiar with the knowledge of one skilled in the art of molecular biology, and peptide structure analysis and synthesis, as of the July 31, 1997, filing date of PCT application PCT/NL97/00449.

I have reviewed the Office Action dated July 16, 2001, in the above-identified application, in part, and specifically the Examiner's rejection of the claims 1-7, 13 and 15-30 under 35 U.S.C. §112, first paragraph, as being non-enabled.

In order to demonstrate that the skilled artisan would have been able to make and/or use the invention commensurate in scope with the pending claims, using routine trial and error experiments, as of July 31, 1997, I further declare and state:

I. Each peptide recited within the pending claims forms an alpha-helix

The first portion of the Examiner's rejection of claims 1-7, 13 and 15-30 as being non-enabled is the Examiner's assertion that the specification, while being enabled for the eight recited peptides (BP1, BP2, BP2.3, BP2.4, BP2.5, BP1.1, BP2.1 and BP2.2[1]) which comprise at least 12 amino acids and are amphipathic, cationic and form a stable alpha-helix, does not provide enablement for all peptides of the consensus formula R^1-R^2-A-B-(A-B-C-A)$_m$-(C)$_n$-R^3 which comprise at least 12 amino acids and are amphipathic, cationic and form a stable alpha-helix.

In particular, the Examiner asserts that "there is no disclosure or description of any data indicating peptides of formula (R^1-R^2-A-B-(A-B-C-A)$_m$-(C)$_n$-R^3) have alpha-helix structures." Further, the Examiner states that "it is known that secondary structures such as alpha-helix, beta-sheet or random coil of protein or peptides can be predicted using the scheme of Chou and

[1] While the Examiner includes peptides BP1.1, BP2.1 and BP2.2 as peptides that are amphipathic, cationic and form a stable alpha-helix, it is asserted that these three peptides do not, in fact, have such characteristics and were utilized in the present application as negative controls.

Fasman with the secondary structure confirmation parameters of amino acid residues in the sequence." The Examiner then states actual measurement of the structure of peptides encompassed within the consensus formula would be required because not all members of the consensus formula form an alpha-helix. The Examiner gives a specific example of a peptide alleged to instead form a beta-pleated sheet.

The Examiner does not question whether the skilled artisan would be able to determine if a given peptide, falling within the scope of the consensus formula, would be amphipathic and cationic.

In response, it is noted that the Examiner apparently uses the scheme of Chou and Fasman to analyze the predicted structure of the peptide she asserts would form a beta-pleated sheet. However, the method of Chou and Fasman was published in 1974 (*Biochemistry* 13:211-222), while the relevant invention date for the instant application was 1997, i.e., 23 years later. In the intermediate period, large numbers of more accurate models were developed and are now used as a matter of routine by persons skilled in the art of peptide structure analysis and synthesis worldwide.

Attached hereto are three printouts (Exhibits 1-3), disclosing a number of the currently accepted methods for peptide modeling, and the dates on which they were published.

Using any of a number of these currently accepted models, it is clear that the peptide recited by the Examiner in the Office Action (Gly-Arg-Tyr-Arg-Ile-Tyr-Arg-Arg-Ile-Tyr-Arg-Arg-Tyr-Ile-Arg-Ile-Ile-Ile-Gly, hereinafter "the EP peptide") does <u>not</u> form a beta-pleated sheet, but instead forms an alpha-helix, as recited in the pending claims. In addition, the skilled artisan

using the currently accepted models would understand that all peptides encompassed within the consensus formula form an alpha-helix, and none of them form a beta-pleated sheet.

To further prove this point, a peptide structural analysis has been performed, by me or under my supervision, with the EP peptide suggested by the Examiner as having a beta-sheet structure, as well as on the BP2 peptide that is encompassed within the consensus formula. The analyses were performed using a number of the modern methodologies, recited below, which are better equipped to predict structure, as these models take into account the structures in a natural environment. Each of the methods revealed that the EP peptide, suggested by the Examiner as having a beta-sheet structure, does not exhibit such a structure, but instead exhibits an alpha-helix structure. Thus, the peptide suggested by the Examiner as being an example of a peptide within the scope of the consensus structure, but not having the characteristics of the proteins recited in the claims, on the contrary is a peptide according to the invention with the required alpha-helix structure.

The methodologies used were SOPMA, HNN and PSIPRED (Exhibits 1-3). Exhibits 4.1, 4.2, 5.1, 5.2 and 6 illustrate the results. The results revealed in these documents are:

4.1)	**SOPMA** - Peptide example of Examiner	alpha helix 84.21% extended strand 0% beta turn 0%
4.2)	**SOPMA** - BP2 peptide	alpha helix 84.21% extended strand 0% beta turn 0%

5.1)	**HNN** - Peptide example of Examiner	alpha helix 63.16% extended strand 21.05% beta turn 0%
5.2)	**HNN** - BP2 peptide	alpha helix 78.95% extended strand 0% beta turn 0%
6)	**PSIPRED** - Peptide example of Examiner	alpha helix
6)	**PSIPRED** - Examples of consensus formula	alpha helix

Thus, quite clearly the example from the present application (BP2) and the EP peptide of the Examiner provided alpha-helical structures according to the prediction.

Indeed, all of the peptides according to the invention adopt alpha-helical structures and this, in fact, is the case according to all of the models analyzed. Contrary to the Examiner's suggestion, none of the peptides according to the invention, applying the models generally used in the field as of July 31, 1997, form a beta-sheet. Thus, there is no requirement for *de novo* experimentation of the skilled person on any of the peptides falling within the structural definition of the claim (i.e., R^1-R^2-A-B-(A-B-C-A)$_m$-(C)$_n$-R^3) to find which will adopt an alpha-helical structure. The formation of an alpha-helix is inherent in peptides encompassed within the structural formula according to claim 1. It is, in fact, already inherent in peptides having the structural formula wherein R_1, R_2 and R_3 are absent.

Thus, as shown herein, it is clear the ordinarily-skilled artisan would be enabled by the disclosure of the present invention to make a peptides comprising at least 12 amino acids, and that are amphipathic, cationic and form stable alpha-helices. Indeed, each of the peptides encompassed within the consensus formula recited in claim 1 are amphipathic, cationic and form stable a stable alpha-helix.

II. The specification enables the skilled artisan to use each of the peptides encompassed within the consensus formula in each of the uses recited in the claims

The second portion of the Examiner's rejection of claims 1-7, 13 and 15-30 as being non-enabled is based on the Examiner's assertion that claims 15-27 are non-enabled because "the specification has not demonstrated a pharmaceutical composition comprising any peptide of formula (R^1-R^2-A-B-(A-B-C-A)$_m$-(C)$_n$-R^3) being used for treating fungus, virus or parasite infection, or treating topic and systemic tumors, inflammation or septic shock, or the treatment being prophylactic."

The Examiner does, however, admit that the specification discloses peptides such as BP1, BP2, BP2.1 or BP2.2 as having antibacterial activity against Gram-positive or Gram-negative bacteria.[2] In addition, it is noted that peptides BP2.3, BP2.4 and BP2.5 also exhibit antibacterial activity (*see*, page 15, lines 16-18, of the specification).

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[2] While the Examiner states at page 5, lines 21-22, that the BP2.1 and BP2.2 peptides have antibacterial activity, it is asserted that these two peptides were used as control peptides (they are not compounds within the consensus sequence of claim 1) and they do not, in fact, exhibit antibacterial activity (*see, e.g.*, page 15, lines 5-18, of the specification).

Due to the shared characteristics of the peptides of the consensus formula, and the results with the experimental peptides reported in the specification, the skilled artisan would expect that each of the peptides of the consensus formula function for the purposes recited in the claims.

In addition, presented herein is experimental evidence from experiments conducted by me or under my supervision, in mice, demonstrating that peptides of the consensus formula can be used for each of the purposes recited in the claims relating to, the treatment of infections by bacteria, inflammation or septic shock, and as a prophylactic.

As outlined in the background of the invention, bacterial membrane components or lipopolysaccharides (LPS) are released during the course of an infection in the host. During acute infection large amounts of these endotoxins in the systemic circulation activate the cellular immune system to produce high levels of inflammatory molecules or cytokines, which have profound effects on the blood vessels and the organs of the body. Persistent stimulation of the cellular defense system by these bacterial components therefore leads to an ongoing systemic inflammation, which is maintained by the high cytokine concentrations. This systemic inflammatory response syndrome (SIRS) underlies serious clinical symptoms such as sepsis, septic shock, inadequate organ perfusion, multiple organ failure and mortality. The degree of severity of the clinical symptoms is related to the cytokine levels which are directly proportional to the amount of endotoxin and hence the number of bacteria present in the circulation. An *in vivo* murine model of lethal endotoxemia that is induced by administration of purified LPS to prove the prophylactic effect of the peptides according to the invention has been described in the patent application (p. 20, line 24 - p. 21, line 22; Fig. 13). In addition, an *in vivo* murine model

of lethal peritonitis induced by the administration of live bacteria to prove the efficacy of the BP2 peptide according to the invention as a therapeutic in the treatment of this condition, has been described in the patent application (p. 21, lines 9-22; Fig. 14). Supplementary data relating to the number of circulating bacteria and cytokine levels (TNF-α) during the course of lethal peritonitis in control and peptide treated groups of mice are illustrated in Exhibit 7. These results indicate that the BP2 peptide (SEQ ID NO: 2) according to the invention is capable of:

1) killing the invading bacteria, evident by reduction of colony forming units (CFU), in the peritoneum and blood,

2) neutralizing the inflammatory potential of LPS, illustrated by markedly reduced TNF-α levels in the peritoneum and blood, thereby preventing the onset of systemic inflammation and septic shock, and

3) increasing survival in experimental animals when the peptide is administered after a lethal challenge of live bacteria in a therapeutic manner.

Taken together, experimental evidence is provided, demonstrating that the peptides of the consensus formula can be used for each of the purposes recited in the claims relating to the treatment of infections by bacteria, the prevention of inflammation or septic shock, and as a prophylactic.

It is well known by a person skilled in the art that the biological activity of antimicrobial peptides in general is not limited to bacterial targets. Selected peptides have previously been shown prior to July 31, 1997, to have a variety of potentially clinically useful activities including

antiviral, antiparasitic, antifungal and antitumor activities (*see* Hancock, R.E.W., The Lancet Infectious Diseases, 2001. Vol. 1, pg 156-164 for a recent review and the references therein).

In addition, presented herein is experimental evidence from preliminary *in vitro* experiments conducted by me or under my supervision, demonstrating that peptides of the consensus formula can be used for each of the purposes recited in the claims relating to the treatment of infections by viruses and parasites, or the treatment of local and systemic tumors.

Antiviral activity of a peptide according to the consensus formula is presented in Exhibit 8. A dose-dependent reduction of HIV-infected peripheral blood mononuclear cells (PBMC) by the BP1 peptide at concentrations was evident. At 1 μM the BP1 effected a 50% reduction of viral replication. At 5 μM the BP1 as well as the BP2 peptide (data not shown) effected a 75% reduction of virus particles. These results indicate that peptides according to the consensus formula possess potent antiviral activity.

Antiparasitic activity of a peptide according to the consensus formula is presented in Exhibit 9. BP2 peptide specifically targets *Plasmodium falciparum* infected human erythrocytes and effects a dose-dependent reduction in the numbers of infected red blood cells. The peptide at the highest concentration tested (20 μg/ml) effected a >90% reduction of infected cells, evident by the almost complete absence of late Trophoblast and Schizont cells representative of the late stages in the *Plasmodium* life-cycle.

Antitumor activity of a peptide according to the consensus formula against two murine transformed cell lines is presented in Exhibit 10. The IC_{50} of the BP2 peptide was found to be

6 μg/ml against murine myeloma P388 cells and approximately 17 μg/ml against the murine fibroblast L929 cell line.

Antifungal activity of peptides according to the consensus formula have not specifically been tested, but similarity in composition and structural features (cationicity, α-helicity and amphipathicity) with other antimicrobial peptides known to possess activity against fungi such as *Candida albicans*, enables a person skilled in the art to anticipate this activity with reasonable confidence.

III. Each peptide encompassed by the consensus formula is effective in the treatment of humans

The third portion of the Examiner's rejection of claims 1-7, 13 and 15-30 as being non-enabled is based on the Examiner's assertion that claims 28-30 are non-enabled because "there is no data [demonstrating] the treatment of microbial infection using the peptide of formula (R^1-R^2-A-B-(A-B-C-A)$_m$-(C)$_n$-R^3) being effective in humans." The Examiner states that the only experimental data disclosed is that of using the peptide BP2 to treat mice. The Examiner further states that the specification does not demonstrate how to predict the expected effects of other peptides encompassed by the consensus formula.

Contrary to the Examiner's position, the skilled person would not doubt that the extrapolation of the results of the tests from the murine models would be applicable to humans. Indeed, murine models of human illnesses are now commonly accepted, and the results from testing in mice is often directly applicable to humans.

Mice represent the primary species used in research, comprising 67% of all animals used in biomedical research and testing. Their short life span, proclivity for reproduction, known genetic background, and minimal expense for purchase and maintenance have made them a desirable animal model. Mice are used in studies involving aging, behavioral research, bioassay and pharmacological screening, chemical mutagenesis and carcinogenesis, convulsive disorders, diabetes and obesity, embryology, immunology, infectious disease research (bacterial, fungal, parasitic, viral), naturally occurring neoplasias, hematopoietic system, mammary tissue, male and female reproductive systems, urinary, endocrine, respiratory, digestive, musculoskeletal, nervous, cardiovascular, and integumentary systems, and ophthalmic research.

For example, B. E. Rollin *et al.*, discuss how mice provide effective models for demonstrating the ability of novel pharmacological agents to be used in the treatment of human genetic and developmental defects, neoplastic disease, metabolic and nutritional disease, degenerative disease and infectious disease (*The Experimental Animal in Biomedical Research, vols. I (1990) and II (1995), CRC Press, Boca Raton, FL*).

Moreover, included herewith are experimental results from experiments conducted by me or under my supervision, showing the efficacy of using peptides of the consensus formula in clearance of bacteria from human whole blood samples.

The antibiotic properties of BP2 (SEQ ID NO:2) against the encapsulated serum-resistant *E. coli* 018:K1:H7 Bort strain in human whole blood (*ex vivo*) is shown in Exhibit 11. The IC_{50} of BP2 against 10^6 CFU/ml was found to be 2.5 μg/ml (1 μM). Time-course analysis with BP2 at 5 μg/ml (2 μM) showed a 95% reduction in the number of viable bacteria after 10 min

increasing to >99% at 1h. These results illustrate that the BP2 peptide encompassed within the consensus formula has potent antibacterial activity against a clinically relevant bacterial pathogen in a complex physiological environment such as human whole blood. In addition, the anti-endotoxin or anti-inflammatory property of the BP2 peptide towards the cytokine-inducing properties of purified LPS in undiluted human whole blood has previously been described in the patent application (p. 20, lines 13-22; Fig. 12). In summary, taken together with the pre-clinical animal data these indications are sufficient for a person skilled in the art to conclude that the peptides according to the invention have a high therapeutic potential for use in the management of human infectious disease.

Finally, I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: January 14, 2002

Name: Philip R. Abraham

UK HGMP RESOURCE CENTRE Funded by the MRC

Welcome to the GenomeWeb Protein Secondary Structure

Search for: Search

EXHIBIT 1

These are a collection of protein secondary structure analysis and information sites.

- PSIpred - Prediction of secondary structure from multiple sequences
- SAM-T99
- BCM Protein Secondary Structure Prediction
- Jnet - a neural network protein secondary structure prediction method
- Jpred - A consensus method for protein secondary structure prediction
- Prof (son of DSC) Secondary Structure Prediction
- Predator
- Nnpredict server
- SOPMA
- PredictProtein - sequence analysis and structure prediction
- HMMTOP - predict transmembrane helices and topology
- SPLIT (Membrane Protein Secondary Structure Prediction)
- DAS (Prediction of transmembrane alpha-helices in prokaryotic membrane proteins)
- SOSUI - Secondary Structure Prediction of Membrane Proteins
- TopPred - Topology prediction of membrane proteins
- TMpred - prediction of transmembrane regions and orientation
- Coils - prediction of coiled coil regions
- Paircoil
- SignalP - predicts signal peptides of secretory proteins
- Sigfind - Signal Peptide Prediction Server (Human)
- ChloroP - Chloroplast Transit Peptide Prediction
- Helix-Turn-Helix (HTH)

Detailed information on the above options

PSIpred - Prediction of secondary structure from multiple sequences
PSIpred - Prediction of secondary structure from multiple sequences

MEMSAT 2 - Prediction of transmembrane topology from multiple sequences

GenTHREADER - Fast and reliable protein fold recognition

SAM-T99
The best 2ry structure predictor at CASP3 was clearly Jones's PSIPRED.

A close second was this predictor. They have since improved thier predictor considerably. They hope to beat PSIPRED at CASP4 with this predictor. Currently, this predictor is about 77-78% correct, and does a good job of knowing when it is inaccurate.

BCM Protein Secondary Structure Prediction
This provides a rich set of programs for protein secondary structure determination.

- Coils - prediction of coiled coil regions
- nnPredict - uses a 2 layer neural network
- PSSP / SSP - segment-oriented prediction
- PSSP / NNSSP - nearest-neighbor prediction
- SAPS - statistical analysis of protein sequences
- TMpred - transmembrane region and orientation prediction
- PHDsec - profile network method
- PSA - for single domain globular proteins
- SOPM - self optimized prediction method
- SSPRED - with residue exchange statistics
- Swiss-Model - from alignment to crystallographic data

Jnet - a neural network protein secondary structure prediction method
Jnet is a neural network prediction algorithm that works by applying multiple sequence alignments, alongside PSIBLAST and HMM profiles. Consensus techniques are applied that predict the final secondary structure more accurately. It was written as part of a continuing study to improve protein secondary structure prediction. Jnet can also predict 2 state solvent exposure at 25, 5 and 0% relative exposure. Positions where the different prediction methods do not agree are marked as no jury positions. A separate network is applied for these positions, which improves the cross-validated accuracy. A reliability index indicates which residues are predicted with a high confidence.

Jpred - A consensus method for protein secondary structure prediction
Jpred takes either a protein sequence or mulitply aligned protein sequences, and predicts secondary structure. It works by combining a number of modern, high quality prediction methods to form a consensus.

Jpred runs DSC, PHD, PREDATOR and NNSSP to build it's consensus prediction, but predictions from older algorithms Mulpred and Zpred are also included in the final output.

The consensus method has been shown, to be on average more accurate than any of the component methods, by ca. 1%. However the strength of this server lies in the fact that it leaves the final decision to the user who can use the supplied coloured HTML and Java viewer to decide where the best or most sensible consensus may be.

Prof (son of DSC) Secondary Structure Prediction
Submit a single amino acid sequence for secondary structure prediction

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Predator
Protein secondary structure prediction from single sequence or from a set of sequences.

PREDATOR takes as input a sequence file in FASTA, MSF or CLUSTAL format containing one or many protein sequences. By default, the prediction will be made for the first sequence in the set.

Nnpredict server
nnpredict is a program that predicts the secondary structure type for each residue in an amino acid sequence. The basis of the prediction is a two-layer, feed-forward neural network.

nnpredict takes as input a sequence consisting of one-letter amino acid codes (A C D E F G H I K L M N P Q R S T V W Y) (NOTE: B and Z are not recognized as valid amino acid codes) or three-letter amino acid codes separated by spaces (ALA CYS ASP GLU PHE GLY HIS ILE LYS LEU MET ASN PRO GLN ARG SER THR VAL TRP TYR). The output is a secondary structure prediction for each position in the sequence. Multiple-chain proteins can be predicted either in pieces, or as a single sequence, with a '!' character between chains.

SOPMA
SOPMA (Self Optimized Prediction Method from Alignment) is a package to make secondary structure predictions of proteins.

PredictProtein - sequence analysis and structure prediction
An automatic service for protein database searches and the prediction of aspects of protein structure.

Database searches:

- generation of multiple sequence alignments (MaxHom)
- detection of functional motifs (PROSITE)
- detection of composition-bias (SEG)
- detection of protein domains (PRODOM)
- fold recognition by prediction-based threading (TOPITS)

Predictions of:

- secondary structure (PHDsec, and PROFsec)
- residue solvent accessibility (PHDacc, and PROFacc)
- transmembrane helix location and topology (PHDhtm, PHDtopology)
- protein globularity (GLOBE)
- coiled-coil regions (COILS)
- cysteine bonds (CYSPRED)
- structural switching regions (ASP)

Evaluation of secondary structure prediction accuracy (EvalSec)

- See also: EVA : an automatic evaluation of prediction methods

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HMMTOP - predict transmembrane helices and topology
HMMTOP is an automatic server for predicting topology of transmembrane proteins. The method is based on the hypothesis that topology is determined by the maximum divergence of the amino acid distributions of the various structural parts in membrane proteins.

SPLIT (Membrane Protein Secondary Structure Prediction)
The purpose of this server is to predict the transmembrane (TM) secondary structures of membrane proteins, using the method of preference functions.

DAS (Prediction of transmembrane alpha-helices in prokaryotic membrane proteins)
The so-called Dense Alignment Surface (DAS) method was introduced in an attempt to improve sequence alignments in the G-protein coupled receptor family of transmembrane proteins. We have now generalized this method to predict transmembrane segments in any integral membrane protein. DAS is based on low-stringency dot-plots of the query sequence against a collection of non-homologous membrane proteins using a previously derived, special scoring matrix.

SOSUI - Secondary Structure Prediction of Membrane Proteins
The SOSUI system is a useful tool for secondary structure prediction of membrane proteins from a protein sequence. The basic idea of prediction in this system is based on the physicochemical properties of amino acid sequences such as hydrophobicity and charges. The system deals with three types of prediction: discrimination of membrane proteins from soluble one, prediction of existence of transmembrane helices and determination of transmembrane helical regions. The accuracy of this system, discrimination of membrane proteins, existence of transmembrane helices and transmembrane helical regions, are about 99%, 96% and 85% respectively.

TopPred - Topology prediction of membrane proteins
A new, simple method for predicting transmembrane segments in integral membrane proteins. It is based on low-stringency dot-plots of the query sequence against a collection of non-homologous membrane proteins using a previously derived scoring matrix. This so-called dense alignment surface (DAS) method is shown to perform on par with earlier methods that require extra information in the form of multiple sequence alignments or the distribution of positively charged residues outside the transmembrane segments, and thus improves prediction abilities when only single-sequence information is available or for classes of membrane proteins that do not follow the 'positive inside' rule.

TMpred - prediction of transmembrane regions and orientation
This program tries to find putative transmembrane domains in proteins and also speculates on the possible orientation of these segments. For its scoring, it uses a combination of multiple weight-matrices that have been extracted from a statistical analysis of TMbase, a collection of all annotated transmembrane proteins present in SwissProt.

Coils - prediction of coiled coil regions
This program predicts (2 stranded) coiled coil regions in proteins by the Lupas-algorithm.

Paircoil
The Paircoil program predicts the location of coiled-coil regions in amino acid sequences.

SignalP - predicts signal peptides of secretory proteins
SignalP predicts signal peptides of secretory proteins. For cleaved signal peptides, the precise location of the cleavage site in the amino acid sequence is predicted. The prediciton is optimised for three different types of organisms: Gram-positive prokaryotes, Gram-negative prokaryotes, and eukaryotes. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks.

SigFind - Signal Peptide Prediction Server (Human)
This software (SIGFIND) predicts signal peptides at the start of protein sequences. A novel neural network learning algorithm is used for prediction.

References

EXHIBIT 2

1

BLAST

Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.
Nucleic Acids Res. 1997 Sep 1;25(17):3389-3402
Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ
National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. altschul@ncbi.nlm.nih.gov

The BLAST programs are widely used tools for searching protein and DNA databases for sequence similarities. For protein comparisons, a variety of definitional, algorithmic and statistical refinements described here permits the execution time of the BLAST programs to be decreased substantially while enhancing their sensitivity to weak similarities. A new criterion for triggering the extension of word hits, combined with a new heuristic for generating gapped alignments, yields a gapped BLAST program that runs at approximately three times the speed of the original. In addition, a method is introduced for automatically combining statistically significant alignments produced by BLAST into a position-specific score matrix, and searching the database using this matrix. The resulting Position-Specific Iterated BLAST (PSI-BLAST) program runs at approximately the same speed per iteration as gapped BLAST, but in many cases is much more sensitive to weak but biologically relevant sequence similarities. PSI-BLAST is used to uncover several new and interesting members of the BRCT superfamily.

CLUSTALW

CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.
Nucleic Acids Res 1994 Nov 11;22(22):4673-4680
Thompson JD, Higgins DG, Gibson TJ
European Molecular Biology Laboratory, Heidelberg, Germany.

The sensitivity of the commonly used progressive multiple sequence alignment method has been greatly improved for the alignment of divergent protein sequences. Firstly, individual weights are assigned to each sequence in a partial alignment in order to down-weight near-duplicate sequences and up-weight the most divergent ones. Secondly, amino acid substitution matrices are varied at different alignment stages according to the divergence of the sequences to be aligned. Thirdly, residue-specific gap penalties and locally reduced gap penalties in hydrophilic regions encourage new gaps in potential loop regions rather than regular secondary structure. Fourthly, positions in early alignments where gaps have been opened receive locally reduced gap penalties to encourage the opening up of new gaps at these positions. These modifications are incorporated into a new program, CLUSTAL W which is freely available.

Coiled-coil prediction

Predicting coiled coils from protein sequences.
Science 1991 May 24;252(5010):1162-1164
Lupas A, Van Dyke M, Stock J
Department of Molecular Biology, Princeton University, NJ 08544.

The probability that a residue in a protein is part of a coiled-coil structure was assessed by comparison of its flanking sequences with sequences of known coiled-coil proteins. This method was used to delineate coiled-coil domains in otherwise globular proteins, such as the leucine zipper domains in transcriptional regulators, and to predict regions of discontinuity within coiled-coil structures, such as the hinge region in myosin. More than 200 proteins that probably have coiled-coil domains were identified in GenBank, including alpha- and beta-tubulins, flagellins, G protein beta subunits, some bacterial transfer RNA synthetases, and members of the heat shock protein (Hsp70) family.

DPM

An algorithm for protein secondary structure prediction based on class prediction.
Protein Eng 1987 Aug;1(4):289-294
Deleage G, Roux B
Laboratoire de Physico-Chimie Biologique, LBTM-CNRS UM 24, Universite Claude Bernard, Villeurbanne, France.

An algorithm has been developed to improve the success rate in the prediction of the secondary structure of proteins by taking into account the predicted class of the proteins. This method has been called the 'double prediction method' and consists of a first prediction of the secondary structure from a new algorithm which uses parameters of the type described by Chou and Fasman, and the prediction of the class of the proteins from their amino acid composition. These two independent predictions allow one to optimize the parameters calculated over the secondary structure database to provide the final prediction of secondary structure. This method has been tested on 59 proteins in the database (i.e. 10,322 residues) and yields 72% success in class prediction, 61.3% of residues correctly predicted for three states (helix, sheet and coil) and a good agreement between observed and predicted contents in secondary structure.

DSC

Identification and application of the concepts important for accurate and reliable protein secondary structure prediction
Protein Sci 1996 Nov;5(11):2298-310
King RD, Sternberg MJ
Biomolecular Modelling Laboratory, Imperial Cancer Research Fund, London, United Kingdom.

A protein secondary structure prediction method from multiply aligned homologous sequences is presented with an overall per residue three-state accuracy of 70.1%. There are two aims: to obtain high accuracy by identification of a set of concepts important for prediction followed by use of linear statistics; and to provide insight into the folding process. The important concepts in secondary structure prediction are identified as: residue conformational propensities, sequence edge effects, moments of hydrophobicity, position of insertions and deletions in aligned homologous sequence, moments of conservation, auto-correlation, residue ratios, secondary structure feedback effects, and filtering. Explicit use of edge effects, moments of conservation, and auto-correlation are new to this paper. The relative importance of the concepts used in prediction was analyzed by stepwise addition of information and examination of weights in the discrimination function. The simple and explicit structure of the prediction allows the method to be reimplemented easily. The accuracy of a prediction is predictable a priori. This permits evaluation of the utility of the prediction: 10% of the chains predicted were identified correctly as having a mean accuracy of > 80%. Existing high-accuracy prediction methods are "black-box" predictors based on complex nonlinear statistics (e.g., neural networks in PHD: Rost & Sander, 1993a). For medium- to short-length chains (> or = 90 residues and < 170 residues), the prediction method is significantly more accurate ($P < 0.01$) than the PHD algorithm (probably the most commonly used algorithm). In combination with the PHD, an algorithm is formed that is significantly more accurate than either method, with an estimated overall three-state accuracy of 72.4%, the highest accuracy reported for any prediction method.

DSSP

Dictionary of protein secondary structure : pattern recognition of hydrogen-bonded and geometrical features
Biopolymers 1983, 22: 2577-2637
Kabsch W & Sander C

FASTA

Searching protein sequence libraries: comparison of the sensitivity and selectivity of the Smith-Waterman and FASTA algorithms.
PNAS (1988) 85:2444-2448
Pearson WR
Department of Biochemistry, University of Virginia, Charlottesville 22908.

The sensitivity and selectivity of the FASTA and the Smith-Waterman protein sequence comparison algorithms were evaluated using the superfamily classification provided in the National Biomedical Research Foundation/Protein Identification Resource (PIR) protein sequence database. Sequences from each of the 34 superfamilies in the PIR database with 20 or more members were compared against the protein sequence database. The similarity scores of the related and unrelated sequences were determined using either the FASTA program or the Smith-Waterman local similarity algorithm. These two sets of similarity scores were used to evaluate the ability of the two comparison algorithms to identify distantly related protein sequences. The FASTA program using the ktup = 2 sensitivity setting performed as well as the Smith-Waterman algorithm for 19 of the 34 superfamilies. Increasing the sensitivity by setting ktup = 1 allowed FASTA to perform as well as Smith-Waterman on an additional 7 superfamilies. The rigorous Smith-Waterman method performed better than FASTA with ktup = 1 on 8 superfamilies, including the globins, immunoglobulin variable regions, calmodulins, and plastocyanins. Several strategies for improving the sensitivity of FASTA were examined. The greatest improvement in sensitivity was achieved by optimizing a band around the best initial region found for every library sequence. For every superfamily except the globins and immunoglobulin variable regions, this strategy was as sensitive as a full Smith-Waterman. For some sequences, additional sensitivity was achieved by including conserved but nonidentical residues in the lookup table used to identify the initial region.

Improved tools for biological sequence comparison.
Pearson WR, Lipman DJ
Department of Biochemistry, University of Virginia, Charlottesville 22908.

We have developed three computer programs for comparisons of protein and DNA sequences. They can be used to search sequence data bases, evaluate similarity scores, and identify periodic structures based on local sequence similarity. The FASTA program is a more sensitive derivative of the FASTP program, which can be used to search protein or DNA sequence data bases and can compare a protein sequence to a DNA sequence data base by translating the DNA data base as it is searched. FASTA includes an additional step in the calculation of the initial pairwise similarity score that allows multiple regions of similarity to be joined to increase the score of related sequences. The RDF2 program can be used to evaluate the significance of similarity scores using a shuffling method that preserves local sequence composition. The LFASTA program can display all the regions of local similarity between two sequences with scores greater than a threshold, using the same scoring parameters and a similar alignment algorithm; these local similarities can be displayed as a "graphic matrix" plot or as individual alignments. In addition, these programs have been generalized to allow comparison of DNA orprotein sequences based on a variety of alternative scoring matrices.

GOR I

Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins.
J Mol Biol 1978 Mar 25;120(1):97-120
Garnier J, Osguthorpe DJ, Robson B

1) Co-operation between a laboratory interested in developi[redacted] for protein secondary structure prediction methods and a lab[redacted]ested in applying and comparing such methods has led to the development of a simple predictive algorithm.
2) 2) Four-state predictions, in which each residue is unambiguously assigned one conformational state of a-helix, extended chain, reverse turn or coil, predict 49% of residue states correctly (in a sample of 26 proteins) when the overall helix and estended-chain content is not taken into account.
3) When the relative abundances of helix, extended chain, reverse turn and coil observed by λ-ray crystallography are tajen into account, a single constant for each protein and type of conformation can be used to bias the prediction. When predictions are optimized in this way, 63% of all residue states are unambiguously and correctly assigned.
4) By analysing the nature of the bias required, proteins can be classified into helix-rich types, pleated-sheet-rich types, and so on. It is shown that, if the type of protein can be determined even approximately by circular dichroism, 57% of residue states can be correctly predicted without taking into account the X-ray structure. Further, comparable predictions can be obtained if, instead of circular dichroism, preliminary predictions are made to assess the protein type.
5) It is emphasized that the numbers quoted here depend on the method used to assess accuracy, and the algorithm is shown to be at least as good as, and usually superior to, the reported predictions methods assessed in the same way.
6) Ways of further enhancing predictions by the use of additional information from hydrophobic triplets and homologous sequences are also explored. Hydrophobic triplet information does not significantly improve predictive power and it is concluded that this information is used by proteins in the next stage of folding. On the other hand, the use of homologous sequences appears to be very promising.
7) The implication of these results in protein folding is discussed.

GOR III

Further developments of protein secondary structure prediction using information theory. New parameters and consideration of residue pairs.
J Mol Biol 1987 Dec 5;198(3):425-443
Gibrat JF, Garnier J, Robson B
Laboratoire de Biochimie-Physique, INRA, Universite de Paris-Sud, Orsay, France.

We have re-evaluated the information used in the Garnier-Osguthorpe-Robson (GOR) method of secondary structure prediction with the currently available database. The framework of information theory provides a means to formulate the influence of local sequence upon the conformation of a given residue, in a rigorous manner. However, the existing database does not allow the evaluation of parameters required for an exact treatment of the problem. The validity of the approximations drawn from the theory is examined. It is shown that the first-level approximation, involving single-residue parameters, is only marginally improved by an increase in the database. The second-level approximation, involving pairs of residues, provides a better model. However, in this case the database is not big enough and this method might lead to parameters with deficiencies. Attention is therefore given to overcoming this lack of data. We have determined the significant pairs and the number of dummy observations necessary to obtain the best result for the prediction. This new version of the GOR method increases the accuracy of prediction by 7%, bringing the amount of residues correctly predicted to 63% for three states and 68 proteins, each protein to be predicted being removed from the database and the parameters derived from the other proteins. If the protein to be predicted is kept in the database the accuracy goes up to 69.7%.

GOR IV

GOR secondary structure prediction method version IV
Methods in Enzymology 1996 R.F. Doolittle Ed., vol 266, 540-553
Garnier J, Gibrat J-F, Robson B

GOR:The GOR method is based on information theory and was developed by J.Garnier, D.Osguthorpe and B.Robson (J.Mol.Biol.120,97, 1978). The present version, GOR IV, uses all possible pair frequencies within a window of 17 amino acid residues and is reported by J. Garnier, J.F. Gibrat and B.Robson in Methods in Enzymology, vol 266, p 540-553 (1996). After crossvalidation on a data base of 267 proteins, the version IV of GOR has a mean accuracy of 64.4% for a three state prediction (Q3). The program gives two outputs, one eye-friendly giving the sequence and the predicted secondary structure in rows, H=helix, E=extended or beta strand and C=coil; the second gives the probability values for each secondary structure at each amino acid position. The predicted secondary structure is the one of highest probability compatible with a predicted helix segment of at least four residues and a predicted extended segment of at least two residues.

HNN

Combinaison de classifieurs statistiques, Application a la prediction de structure secondaire des proteines
PhD Thesis
Guermeur, Y
Model combination has recently been at the origine of significant improvements in the field of statistical learning, both for regression and pattern recognition tasks. However, fundamental questions have remained virtually untackled. Few criteria have thus been developed to motivate the choice of a specific method, whereas no independent result has been derived in the field of discrimination. This dissertation deals with one of the most commonly used combination techniques: linear regression. We first characterize the regularizing effect of the "stacked regression" method introduced by Breiman. We then study the application of the multivariate linear regression model to the combination of discriminant experts the outputs of which are estimates of th class posterior probabilities. This question is successively considered from the point of view of optimization and complexity control. The latter point involves the computation of generalized Vapnik-Chervonenkis dimensions. The study is followed up with the description of a non parametric method fo Bayes' error rate estimation. Our ensemble method is assessed on an open biological sequence processing problem: the problem of globular protein secondary structure prediction. To perform this discrimination task, we introduce a hierarchical and modular approach in which combination is used at an intermediate level.

Helix-turn-helix DNA-binding motifs prediction

Improved detection of helix-turn-helix DNA-binding motifs in protein sequences.
Nucleic Acids Res 1990 Sep 11;18(17):5019-5026
Dodd IB, Egan JB
Department of Biochemistry, University of Adelaide, Australia.

We present an update of our method for systematic detection and evaluation of potential helix-turn-helix DNA-binding motifs in protein sequences [Dodd, I. and Egan, J. B. (1987) J. Mol. Biol. 194, 557-564]. The new method is considerably more powerful, detecting approximately 50% more likely helix-turn-helix sequences without an increase in false predictions. This improvement is due almost entirely to the use of a much larger reference set of 91 presumed helix-turn-helix sequences. The scoring matrix derived from this reference set has been calibrated against a large protein sequence database so that the score obtained by a sequence can be used to give a practical estimation of the probability that the sequence is a helix-turn-helix motif.

MLRC

Improved Performance in Protein Secondary Structure Prediction by Inhomogeneous Score Combination
Bioinformatics vol. 15 no. 5 1999 pp 413-421
Guermeur Y, Geourjon C, Gallinari P, & Deleage G
Motivation:
In many fields of pattern recognition, combination has proved efficient to increase the generalization performance of individual prediction methods. Numerous systems have been developed for protein secondary structure prediction, based on different principles. Finding better ensemble methods for this task may thus become crucial. In addition, efforts need to be made to help the biologist in the post-processing of the outputs. Results:
An ensemble method has been designed to post-process the outputs of protein secondary structure prediction methods, in order to obtain an improvement of prediction accuracy while generating class posterior probability estimates. Experimental results establish that it can increase the recognition rate of methods that provide inhomogeneous scores, even if their individual prediction successes are largely different. This combination thus contsitutes an help for the biologist, who can use it confidently on top of any set of prediction methods. Furthermore, the resulting estimates can be used in various ways, for instance to determine which residues are predicted with a given high level of reliability. Availability:
Free availability over the internet on the Network Protein Sequence @nalysis (NPS@) WWW server at http://npsa-pbil.ibcp.fr/NPSA/npsa_mlr.html. The method is proposed as the default choice. Contact:
Neural networks and ensemble method : Yann.Guermeur@ens-lyon.fr, server and software : g.deleage@ibcp.fr.

MPSA

MPSA: integrated system for multiple protein sequence analysis with client/server capabilities.
Bioinformatics 2000 Mar;16(3):286-7
Blanchet C, Combet C, Geourjon C, Deleage G
Summary: MPSA is a stand-alone software intended to protein sequence analysis with a high integration level and Web clients/server capabilities. It provides many methods and tools, which are integrated into an interactive graphical user interface. It is available for most Unix/Linux and non-Unix systems. MPSA is able to connect to a Web server (e.g. http://mpsa-pbil.ibcp.fr/) in order to perform large-scale sequence comparison on up-to-date databanks. Availability: Free to academic http://mpsa-pbil.ibcp.fr/ Contact: c.blanchet@ibcp.fr

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Multalin

Multiple sequence alignment with hierarchical clustering.
Nucleic Acids Res 1988 Nov 25;16(22):10881-10890
Corpet F
Laboratoire de Genetique Cellulaire, INRA Toulouse, France.

An algorithm is presented for the multiple alignment of sequences, either proteins or nucleic acids, that is both accurate and easy to use on microcomputers. The approach is based on the conventional dynamic-programming method of pairwise alignment. Initially, a hierarchical clustering of the sequences is performed using the matrix of the pairwise alignment scores. The closest sequences are aligned creating groups of aligned sequences. Then close groups are aligned until all sequences are aligned in one group. The pairwise alignments included in the multiple alignment form a new matrix that is used to produce a hierarchical clustering. If it is different from the first one, iteration of the process can be performed. The method is illustrated by an example: a global alignment of 39 sequences of cytochrome c.

NPS@

NPS@: Network Protein Sequence Analysis
TIBS 2000 March Vol. 25, No 3 [291]:147-150
Combet C., Blanchet C., Geourjon C. and Deléage G.

P-SEA

P-SEA: a new efficient assignment of secondary struc[redacted] alpha trace of proteins.
Comput Appl Biosci 1997 Jun;13(3):291-5
Labesse G, Colloc'h N, Pothier J, Mornon JP

MOTIVATION: The secondary structure is a key element of architectural organization in proteins. Accurate assignment of the secondary structure elements (SSE) (helix, strand, coil) is an essential step for the analysis and modelling of protein structure. Various methods have been proposed to assign secondary structure. Comparative studies of their results have shown some of their drawbacks, pointing out the difficulties in the task of SSE assignment.
RESULTS: We have designed a new automatic method, named P-SEA, to assign efficiently secondary structure from the sole C alpha position. Some advantages of the new algorithm are discussed.
AVAILABILITY: The program P-SEA is available by anonymous ftp: ftp.lmcp.jussieu.fr directory: pub/.

PHD

Prediction of protein secondary structure at better than 70% accuracy.
J Mol Biol 1993 Jul 20;232(2):584-99
Rost B, Sander C
European Molecular Biology Laboratory, Heidelberg, Germany.

We have trained a two-layered feed-forward neural network on a non-redundant data base of 130 protein chains to predict the secondary structure of water-soluble proteins. A new key aspect is the use of evolutionary information in the form of multiple sequence alignments that are used as input instead of single sequences. The inclusion of protein family information in this form increases the prediction accuracy by six to eight percentage points. A combination of three levels of networks results in an overall three-state accuracy of 70.8% for globular proteins (sustained performance). If four membrane protein chains are included in the evaluation, the overall accuracy drops to 70.2%. The prediction is well balanced between alpha-helix, beta-strand and loop: 65% of the observed strand residues are predicted correctly. The accuracy in predicting the content of three secondary structure types is comparable to that of circular dichroism spectroscopy. The performance accuracy is verified by a sevenfold cross-validation test, and an additional test on 26 recently solved proteins. Of particular practical importance is the definition of a position-specific reliability index. For half of the residues predicted with a high level of reliability the overall accuracy increases to better than 82%. A further strength of the method is the more realistic prediction of segment length. The protein family prediction method is available for testing by academic researchers via an electronic mail server.

Combining evolutionary information and neural networks to predict protein secondary structure.
Proteins 1994 May;19(1):55-72
Rost B, Sander C
European Molecular Biology Laboratory, Heidelberg, Germany.

Using evolutionary information contained in multiple sequence alignments as input to neural networks, secondary structure can be predicted at significantly increased accuracy. Here, we extend our previous three-level system of neural networks by using additional input information derived from multiple alignments. Using a position-specific conservation weight as part of the input increases performance. Using the number of insertions and deletions reduces the tendency for overprediction and increases overall accuracy. Addition of the global amino acid content yields a further improvement, mainly in predicting structural class. The final network system has sustained overall accuracy of 71.6% in a multiple cross-validation test on 126 unique protein chains. A test on a new set of 124 recently solved protein structures that have no significant sequence similarity to the learning set confirms the high level of accuracy. The average cross-validated accuracy for all 250 sequence-unique chains is above 72%. Using various data sets, the method is compared to alternative prediction methods, some of which also use multiple alignments: the performance advantage of the network system is at least 6 percentage points in three-state accuracy. In addition, the network estimates secondary structure content from multiple sequence alignments about as well as circular dichroism spectroscopy on a single protein and classifies 75% of the 250 proteins correctly into one of four protein structural classes. Of particular practical importance is the definition of a position-specific reliability index. For 40% of all residues the method has a sustained three-state accuracy of 88%, as high as the overall average for homology modelling. A further strength of the method is greatly increased accuracy in predicting the placement of secondary structure segments.

Physico-chemical profiles

A computer program for predicting protein antigenic determinants.
Mol Immunol 1983 Apr;20(4):483-489
Hopp TP, Woods KR

A computerized method for predicting the locations of protein antigenic determinants is presented, which requires only the amino acid sequence of a protein, and no other information. This procedure has been used to predict the major antigenic determinant of the hepatitis B surface antigen, as well as antigenic sites on a series of test proteins of known antigenic structure [Hopp & Woods (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 3824-3828.] The method is suitable for use in smaller personal computers, and is written in the BASIC language, in order to make it available to investigators with limited computer experience and/or resources. A means of locating multiple antigenic sites on a homologous series of proteins is demonstrated using the influenza hemagglutinin as an example.

A simple method for displaying the hydropathic character of a protein.
J Mol Biol 1982 May 5;157(1):105-132
Kyte J, Doolittle RF

A computer program that progressively evaluates the hydrophilicity and hydrophobicity of a protein along its amino acid sequence has been devised. For this purpose, a hydropathy scale has been composed wherein the hydrophilic and hydrophobic properties of each of the 20 amio acid side-chains is taken into consideration. The scale is based on an amalgran of experimental observations derived from the literature. The program uses a moving-segment approach that continuously determine the average hydropathy within a segment of predetermined length as it advances through the sequence. The consecutive scores are plotted from the amino to the carboxy terminus. At the same time, a midpoint line is printed that corresponds to the grand average of the hydropathy of the amino acid compositions found in most of the sequenced proteins. In the case of soluble, globular proteins there is a remarkable correpondence between the interior portions of their sequence and the regions appearing on the hydrophobic side of the midpoint line, as well as the exterior portions and the regions on the hydrophilic side. The correlation was demonstrated by comparisons between the plotted values and known structures determined by cristallography. In the case of membrane-bound proteins, the portions of their sequences that are located within the lipid bilayer are also clearly delineated by large uninterrupted areas on the hydrophobic side of the midpoint line. As such, the membrane-spannin segments of these proteins can be identified by this procedure. Although the method is not unique and embodies principles that have long been appreciated, its simplicity and its graphic nature make it a very useful tool for the evaluation of protein structures.

Prediction of chain flexibility in proteins
Naturwissens-chaften (1985).72, 212-213
Karplus, P.A. & Schulz, G.E

No summary available yet

New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites.
Biochemistry 1986 Sep 23;25(19):5425-5432
Parker JM, Guo D, Hodges RS

A new set of hydrophilicity high-performance liquid chromatography (HPLC) parameters is presented. These parameters were derived from the retention times of 20 model synthetic peptides, Ac-Gly-X-X-(Leu)3-(Lys)2-amide, where X was substituted with the 20 amino acids found in proteins. Since hydrophilicity parameters have been used extensively in algorithms to predict which amino acid residues are antigenic, we have compared the profiles generated by our new set of hydrophilic HPLC parameters on the same scale as nine other sets of parameters. Generally, it was found that the HPLC parameters obtained in this study correlated best with antigenicity. In addition, it was shown that a combination of the three best parameters for predicting antigenicity further improved the predictions. These predicted surface sites or, in other words, the hydrophilic, accessible, or mobile regions were then correlated to the known antigenic sites from immunological studies and accessible sites determined by X-ray crystallographic data for several proteins.

Structural prediction of membrane-bound proteins.
Eur J Biochem 1982 Nov 15;128(2-3):565-575
Argos P, Rao JK, Hargrave PA

A prediction algorithm based on physical characteristics of the twenty amino acids and refined by comparison to the proposed bacteriorhodopsin structure was devised to delineate likely membrane-buried regions in the primary sequences of proteins known to interact with the lipid bilayer. Application of the method to the sequence of the carboxyl terminal one-third of bovine rhodopsin predicted a membrane-buried helical hairpin structure. With the use of lipid-buried segments in bacteriorhodopsin as well as regions predicted by the algorithm in other membrane-bound proteins, a hierarchical ranking of the twenty amino acids in their preferences to be in lipid contact was calculated. A helical wheel analysis of the predicted regions suggests which helical faces are within the protein interior and which are in contact with the lipid bilayer.

PREDATOR

Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence.
Protein Eng 1996 Feb;9(2):133-142
Frishman D, Argos P
European Molecular Biology Laboratory, Heidelberg, Germany.

Existing approaches to protein secondary structure prediction from the amino acid sequence usually rely on the statistics of local residue interactions within a sliding window and the secondary structural state of the central residue. The practically achieved accuracy limit of such single residue and single sequence prediction methods is 65% in three structural stages (alpha-helix, beta-strand and coil). Further improvement in the prediction quality is likely to require exploitation of various aspects of three-dimensional protein architecture. Here we make such an attempt and present an accurate algorithm for secondary structure prediction based on recognition of potentially hydrogen-bonded residues in a single amino acid sequence. The unique feature of our approach involves database-derived statistics on residue type occurrences in different classes of beta-bridges to delineate interacting beta-strands. The alpha-helical structures are also recognized on the basis of amino acid occurrences in hydrogen-bonded pairs (i,i + 4). The algorithm has a prediction accuracy of 68% in three structural stages, relies only on a single protein sequence as input and has the potential to be improved by 5-7% if homologous aligned sequences are also considered.

PSI-BLAST

See BLAST

Secondary consensus prediction

Protein structure prediction. Implications for the biol
Biochimie 1997 Nov;79(11):681-686
Deleage G, Blanchet C, Geourjon C
Institute of Biology and Chemistry of Proteins, Lyon, France.

Recent improvements in the prediction of protein secondary structure are described, particularly those methods using the information contained into multiple alignments. In this respect, the prediction accuracy has been checked and methods that take into account multiple alignments are 70% correct for a three-state description of secondary structure. This quality is obtained by a 'leave-one out' procedure on a reference database of proteins sharing less than 25% identity. Biological applications such as 'protein domain design' and structural phylogeny are given. The biologist's point of view is also considered and joint predictions are encouraged in order to derive an amino acid based accuracy. All the tools described in this paper are available for biologists on the Web (http://www.ibcp.fr/predict.html).

SIMPA96

An algorithm for secondary structure determination in proteins based on sequence similarity.
FEBS Lett 1986 Sep 15;205(2):303-308
Levin JM, Robson B, Garnier J

A secondary structure prediction algorithm is proposed on the hypothesis that short homologous sequences of amino acids have the same secondary structure tendencies. Comparisons are made with the secondary structure assignments of Kabsch and Sander from X-ray data [(1983) Biopolymers 22, 2577-2637] and an empirically determined similarity matrix which assigns a sequence similarity score between any two sequences of 7 residues in length. This similarity matrix differs in many respects from that of the Dayhoff substitution matrix [(1978) in: Atlas of Protein Sequence and Structure, (Dayhoff, M.O. ed). vol. 5. suppl. 3, pp. 353-358, National Biochemical Research Foundation, Washington, DC]. This homologue method had a prediction accuracy of 62.2% over 3states for 61 proteins and 63.6% for a new set of 7 proteins not in the original data base.

Exploring the limits of nearest neighbour secondary structure prediction.
Protein Eng. (1997),7, 771-776
J. LEVIN.

SIMPA is a nearest neighbour method for predicting secondary structures using a similarity matrix, in its latest version the BLOSUM 62, an optimized similarity threshold, a window of 13 to 17 residues and a database of observed secondary structures. In version simpa96 used here, the database contains circa 300 proteins and the window is 13 residues long. Its crossvalidated accuracy was a Q3 of 67.7% for a single sequence and 72.8% when using multiple alignments of homologous sequences.

Major references:
- J. LEVIN, B. ROBSON, J. GARNIER. An Algorithm for secondary structure determination in proteins based on sequence similarity. FEBS, 205, (1986) 303-308. This describes the basic algorithm.
- J. LEVIN, J. GARNIER. Improvements in a secondary structure prediction method based on a search for local sequence homologies and its use as a model building tool. Biochim. Biophys. Acta, (1988) 955, 283-295. Here the window and threshold are optimized and the results are crossvalidated by jack knife process.
- J. LEVIN. Exploring the limits of nearest neighbour secondary structure prediction. Protein Eng. (1997),7, 771-776 This corresponds to simpa96.

SOPM

SOPM: a self-optimized method for protein secondary structure prediction.
Protein Eng 1994 Feb;7(2):157-164
Geourjon C, Deleage G
Institut de Biologie et de Chimie des Proteines, UPR 412-CNRS, Lyon, France.

A new method called the self-optimized prediction method (SOPM) has been developed to improve the success rate in the prediction of the secondary structure of proteins. This new method has been checked against an updated release of the Kabsch and Sander database, 'DATABASE.DSSP', comprising 239 protein chains. The first step of the SOPM is to buildsub-databases of protein sequences and their known secondary structures drawn from 'DATABASE.DSSP' by (i) making binary comparisons of all protein sequences and (ii) taking into account the prediction of structural classes of proteins. The second step is to submit each protein of the sub-database to a secondary structure prediction using a predictive algorithm based on sequence similarity. The third step is to iteratively determine the predictive parameters that optimize the prediction quality on the whole sub-database. The last step is to apply the final parameters to the query sequence. This new method correctly predicts 69% of amino acids for a three-state description of the secondary structure (alpha helix, beta sheet and coil) in the whole database (46,011 amino acids). The correlation coefficients are C alpha = 0.54, C beta = 0.50 and Cc = 0.48. Root mean square deviations of 10% in the secondary structure content are obtained. Implications for the users are drawn so as to derive an accuracy at the amino acid level and provide the user with a guide for secondary structure prediction. The SOPM method is available by anonymous ftp to ibcp.fr.

SOPMA

SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments.
Comput Appl Biosci 1995 Dec;11(6):681-684
Geourjon C, Deleage G
Institut de Biologie et de Chimie des Proteines, UPR 412-CNRS, Lyon, France.

Recently a new method called the self-optimized prediction method (SOPM) has been described to improve the success rate in the prediction of the secondary structure of proteins. In this paper we report improvements brought about by predicting all the sequences of a set of aligned proteins belonging to the same family. This improved SOPM method (SOPMA) correctly predicts 69.5% of amino acids for a three-state description of the secondary structure (alpha-helix, beta-sheet and coil) in a whole database containing 126 chains of non-homologous (less than 25% identity) proteins. Joint prediction with SOPMA and a neural networks method (PHD) correctly predicts 82.2% of residues for 74% of co-predicted amino acids. Predictions are available by Email to deleage@ibcp.fr or on a Web page (http://www.ibcp.fr/predict.html).

SSEARCH

Identification of common molecular subsequences.
J. Mol. Biol. (1981) 147:195-197
Smith TF, Waterman MS

No summary available yet

STRIDE

Knowledge-based secondary structure assignment
Proteins: structure, function and genetics (1995), 23, 566-579
Frishman D & Argos P

Transmembrane helices prediction

Transmembrane helices predicted at 95% accuracy.
Protein Sci 1995 Mar;4(3):521-33
Rost B, Casadio R, Fariselli P, Sander C
Protein Design Group, EMBL Heidelberg, Germany.

We describe a neural network system that predicts the locations of transmembrane helices in integral membrane proteins. By using evolutionary information as input to the network system, the method significantly improved on a previously published neural network prediction method that had been based on single sequence information. The input data were derived from multiple alignments for each position in a window of 13 adjacent residues: amino acid frequency, conservation weights, number of insertions and deletions, and position of the window with respect to the ends of the protein chain. Additional input was the amino acid composition and length of the whole protein. A rigorous cross-validation test on 69 proteins with experimentally determined locations of transmembrane segments yielded an overall two-state per-residue accuracy of 95%. About 94% of all segments were predicted correctly. When applied to known globular proteins as a negative control, the network system incorrectly predicted fewer than 5% of globular proteins as having transmembrane helices. The methodwas applied to all 269 open reading frames from the complete yeast VIII chromosome. For 59 of these, at least two transmembrane helices were predicted. Thus, the prediction is that about one-fourth of all proteins from yeast VIII contain one transmembrane helix, and some 20%, more than one.

DATABASES

PROSITE

The PROSITE database, its status in 1997.
Nucleic Acids Res. (1997)Jan 1;25(1):217-221
Bairoch A, Bucher P, Hofmann K
Department of Medical Biochemistry, University of Geneva, 1 rue Michel Servet 1211 Geneva 4, Switzerland. bairoch@cmu.unige.ch

The PROSITE database consists of biologically significant patterns and profiles formulated in such a way that with appropriate computational tools it can help to determine to which known family of protein (if any) a new sequence belongs, or which known domain(s) it contains.

SWISS-PROT

The SWISS-PROT protein sequence data bank and its supplement TrEMBL.
Nucleic Acids Res 1997 Jan 1;25(1):31-36
Bairoch A, Apweiler R

Department of Medical Biochemistry, University of Geneva [redacted] el Servet, 1211 Geneva 4, Switzerland. bairoch@cmu.unige.[redacted]

SWISS-PROT is a curated protein sequence database which strives to provide a high level of annotations (such as the description of the function of a protein, structure of its domains, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases. Recent developments of the database include: an increase in the number and scope of model organisms; cross-references to two additional databases; a variety of new documentation files and the creation of TrEMBL, a computer annotated supplement to SWISS-PROT. This supplement consists of entries in SWISS-PROT-like format derived from the translation of all coding sequences (CDS) in the EMBL nucleotide sequence database, except the CDS already included in SWISS-PROT.

Exhibit 3

Secondary Structure Prediction methods

There are now many web servers for structure prediction, here is quick summary:

PSI-pred (PSI-BLAST profiles used for prediction; David Jones, Warwick)

JPRED Consensus prediction (includes many of the methods given below; Cuff & Barton, EBI)

DSC King & Sternberg (this server)

PREDATOR Frischman & Argos (EMBL)

PHD home page Rost & Sander, EMBL, Germany

ZPRED server Zvelebil et al., Ludwig, U.K.

nnPredict Cohen et al., UCSF, USA.

BMERC PSA Server Boston University, USA

SSP (Nearest-neighbor) Solovyev and Salamov, Baylor College, USA.

With no homologue of known structure from which to make a 3D model, a logical next step is to predict secondary structure. Although they differ in method, the aim of secondary structure prediction is to provide the location of alpha helices, and beta strands within a protein or protein family.

Methods for single sequences

Secondary structure prediction has been around for almost a quarter of a century. The early methods suffered from a lack of data. Predictions were performed on single sequences rather than families of homologous sequences, and there were relatively few known 3D structures from which to derive parameters. Probably the most famous early methods are those of Chou & Fasman, Garnier, Osguthorbe & Robson (GOR) and Lim. Although the authors originally claimed quite high accuracies (70-80 %), under careful examination, the methods were shown to be only between 56 and 60% accurate (see Kabsch & Sander, 1984 given below). An early problem in secondary structure prediction had been the inclusion of structures used to derive parameters in the set of structures used to assess the accuracy of the method.

Some good references on the subject:

Early methods on single sequences

Chou, P.Y. & Fasman, G.D. (1974). Biochemistry, 13, 211-222.
Lim, V.I. (1974). Journal of Molecular Biology, 88, 857-872.
Garnier, J., Osguthorpe, D.~J. \& Robson, B. (1978).Journal of Molecular Biology, 120, 97-120.
Kabsch, W. & Sander, C. (1983). FEBS Letters, 155, 179-182. (An assessment of the above methods)

Later methods on single sequences

Deleage, G. & Roux, B. (1987). Protein Engineering , 1, 289-294 (DPM)

Presnell, S.R., Cohen, B.I. & Cohen, F.E. (1992). Biochemistry, 31, 983-993.

Holley, H.L. & Karplus, M. (1989). Proceedings of the National Academy of Science, 86, 152-156.

King, R. & Sternberg, M. J.E. (1990). Journal of Molecular Biology, 216, 441-457.

D. G. Kneller, F. E. Cohen & R. Langridge (1990) Improvements in Protein Secondary Structure Prediction by an Enhanced Neural Network, Journal of Molecular Biology, 214, 171-182. (NNPRED)

Recent improvments

The availability of large families of homologous sequences revolutionised secondary structure prediction. Traditional methods, when applied to a family of proteins rather than a single sequence proved much more accurate at identifying core secondary structure elements. The combination of sequence data with sophisticated computing techniques such as neural networks has lead to accuracies well in excess of 70 %. Though this seems a small percentage increase, these predictions are actually much more useful than those for single sequence, since they tend to predict the core accurately. Moreover, the limit of 70-80% may be a function of secondary structure variation within homologous proteins.

Automated methods

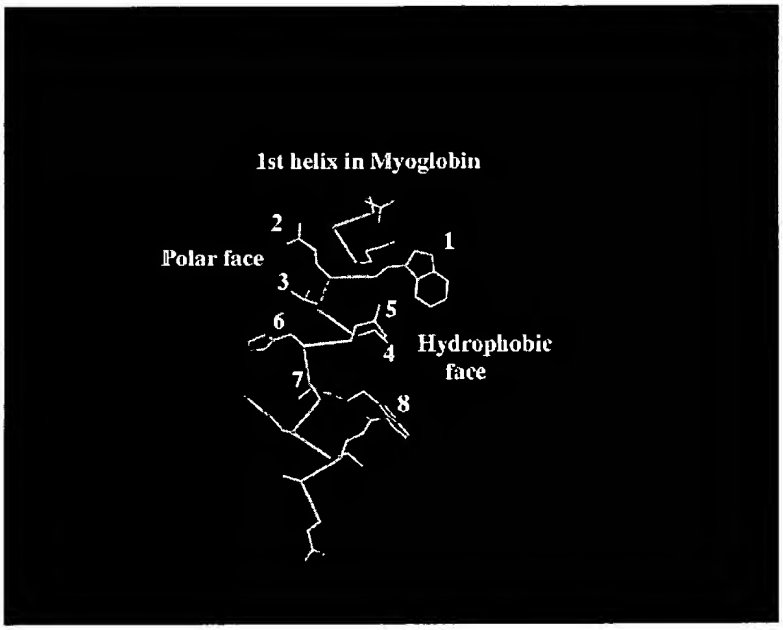
There are numerous automated methods for predicting secondary structure from multiply aligned protein sequences. Some good references on the subject include (the acronyms in parentheses given after each reference refer to the associated WWW servers, given below):

Zvelebil, M.J.J.M., Barton, G.J., Taylor, W.R. & Sternberg, M.J.E. (1987). Prediction of Protein Secondary Structure and Active Sites Using the Alignment of Homologous Sequences Journal of Molecular Biology, 195, 957-961. (ZPRED)
Rost, B. & Sander, C. (1993), Prediction of protein secondary structure at better than 70 % Accuracy, Journal of Molecular Biology, 232, 584-599. PHD)
Salamov A.A. & Solovyev V.V. (1995), Prediction of protein secondary sturcture by combining nearest-neighbor algorithms and multiply sequence alignments. Journal of Molecular Biology, 247,1 (NNSSP)
Geourjon, C. & Deleage, G. (1994), SOPM : a self optimised prediction method for protein secondary structure prediction. Protein Engineering, 7, 157-16. (SOPMA)
Solovyev V.V. & Salamov A.A. (1994) Predicting alpha-helix and beta-strand segments of globular proteins. (1994) Computer Applications in the Biosciences,10,661-669. (SSP)
Wako, H. & Blundell, T. L. (1994), Use of amino-acid environment-depdendent substitution tables and conformational propensities in structure prediction from aligned sequences of homologous proteins. 2. Secondary Structures, Journal of Molecular Biology, 238, 693-708.
Mehta, P., Heringa, J. & Argos, P. (1995), A simple and fast approach to prediction of protein secondary structure from multiple aligned sequences with accuracy above 70 %. Protein Science, 4, 2517-2525. (SSPRED)
King, R.D. & Sternberg, M.J.E. (1996) Identification and application of the concepts important for accurate and reliable protein secondary structure prediction. Protein Sci,5, 2298-2310. (DSC).

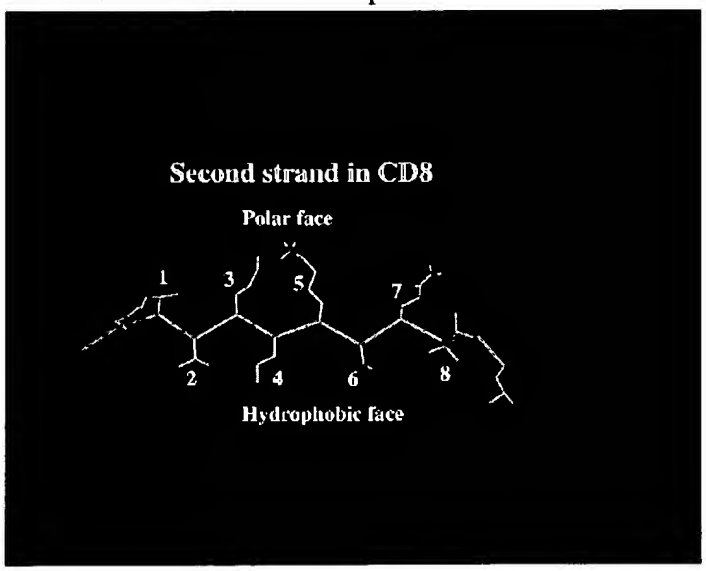
Nearly all of these now run via the world wide web. For individual details, see the papers for the individual methods, or click on the underlined acronyms given after most of the references given above (note that you can also run the methods by going to the approriate WWW site).

Manual intervention

It has long been recognised that patterns of residue conservation are indicative of particular secondary structure types. Alpha helices have a periodicity of 3.6, which means that for helices with one face buried in the protein core, and the other exposed to solvent, will have residues at positions i, i+3, i+4 & i+7 (where i is a residue in an a helix) will lie on one face of the helix. Many alpha helices in proteins are amphipathic, meaning that one face is pointing towards the hydrophobic core and the other towards the solvent. Thus patterns of hydrophobic residue conservation showing the i, i+3, i+4, i+7 pattern are highly indicative of an alpha helix. For example, this helix in myoglobin has this classic pattern of hydrophobic and polar residue conservation (i = 1):

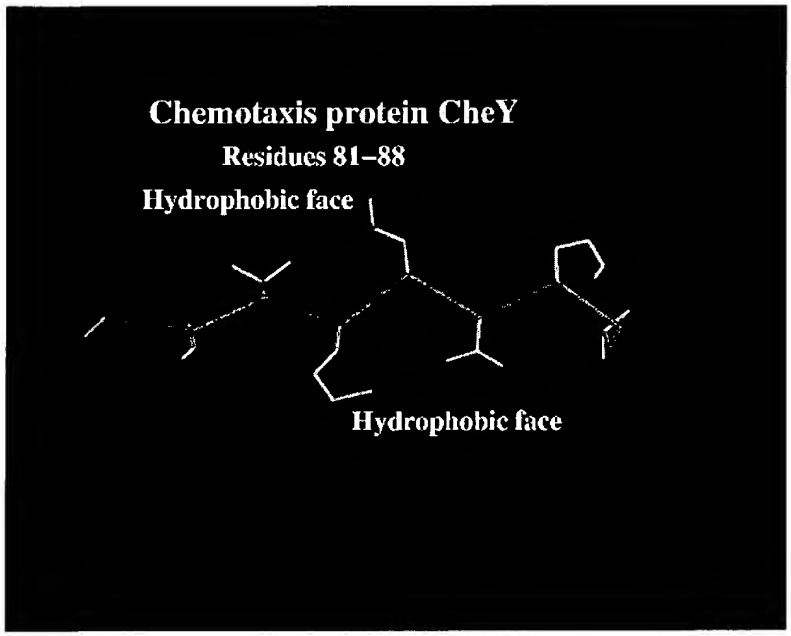


Similarly, the geometry of beta strands means that adjacent residues have their side chains pointing in oppposite directions. Beta strands that are half buried in the protein core will tend to have hydrophobic residues at positions i, i+2, i+4, i+8 etc, and polar residues at positions i+1, i+3, i+5, etc.
For example, this beta strand in CD8 shows this classic pattern:



Beta strands that are completely buried (as is often the case in proteins containing both alpha helices and beta strands) usually contain a run of hydrophobic residues, since both faces are buried in the protein core.

This strand from Chemotaxis protein CheY is a good example:



The principle behind most manual secondary structure predictions is to look for patterns of residue conservation that are indicative of secondary structures like those shown above. It has been shown in numerous successful examples that this strategy often leads to nearly perfect predictions. The work of Barton et al, Nierman & Krischner, Bazan and Benner & co-workers provide good starting points for getting doing this sort of work oneself. Some useful references are:

Recent reviews on the subject (and on secondary structure prediction generally)
See also references therein.

Rost, B., Schneider, R. & Sander, C. (1993), Trends in Biochemical Sciences, 18, 120-123.
Benner, S. A., Gerloff, D. L. & Jenny, T. F. (1994), Science, 265, 1642-1644.
Barton, G. J. (1995), Protein Secondary Structure Prediction, Current Opinion in Structural Biology,5, 372-376.
Russell, R. B. & Sternberg, M. J. E. (1995), Protein Structure Prediction: How Good Are We?, Current Biology, 5, 488-490.

Some guides for predicting structure:

Benner, S. A. (1989), Patterns of divergence in homolgous proteins as indicators of tertiary and quaternary structure, Advances in Enzyme Regulation, 31, 219-236.
Benner, S. A. (1992), Predicting de novo the folded structure of proteins, Current Opinion in Structural Biology, 2, 402-412.

Some particular examples of protein secondary structure predictions:

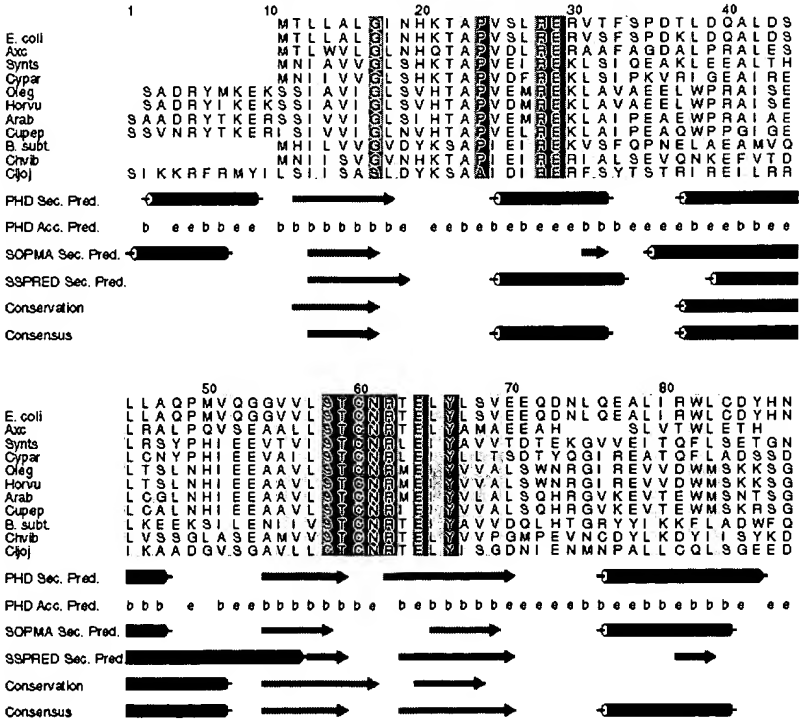
Crawford, I. P., Niermann, T. & Kirschner, K. (1987), Predictions of secondary structure by evolutionary comparison: Application to the alpha subunit of tryptophan synthase, PROTEINS: Structure, Function and Genetics, 1, 118-129.
Bazan, J. F. (1990), Structural Design and Molecular Evolution of a Cytokine Receptor Superfamily,Proceedings of the National Academy of Science, 87, 6934-6938.
Benner, S. A. & Gerloff, D. (1990), Patterns of Divergence in Homologous Proteins and tertiary structure. A prediction of the structure of the catalytic domain of protein kinases, Advances in Enzyme Regulation, 31, 121-181.
Jenny, T. F. & Benner, S. A. (1994) A prediction of the secondary structure of the pleckstrin homology domain, A prediction of the secondary structure of the pleckstrin homology domain, PROTEINS: Structure, Function and Genetics, 20, 1-3.
Benner, S. A., Badcoe, I., Cohen, M. A. and Gerloff, D. L. (1993) Predicted secondary structure for the src

homology 3 domain, Journal of Molecular Biology, 229, 295-305.
Gerloff, D. L., Jenny, T. F., Knecht, L. J., Gonnet, G.H. & Benner, S. A. (1993), The nitrogenase MoFe protein. A secondary structure prediction. FEBS Letters, 318, 118-124.
Gerloff, D. L., Chelvanayagam, G. & Benner, S. A. (1995), A predicted consensus structure for the protein-kinase c2 homology (c2h) domain, the repeating unit of synaptotagmin, PROTEINS: Structure, Function and Genetics, 22, 299-310.
Barton, G. J., Newman, R. H., Freemont, P. F. & Crumpton, M. J. (1991), Amino acid sequence analysis of the annexin super-gene family of proteins, European Journal of Biochemistry, 198, 749-760.
Russell, R. B., Breed, J. & Barton, G. J., (1992) Conservation analysis and secondary structure prediction of the SH2 family of phosphotyrosine binding domains, FEBS Letters, 304, 15-20.
Livingstone, C. D. & Barton, G. J. (1994), Secondary structure prediction from multiple sequence data: Blood clotting factor XII and Yersinia protein tyrosine phosphatase, International Journal of Peptide and Protein Research
Barton, G. J., Barford, D. A. & Cohen, P. T. (1994), European Journal of Biochemsitry, 220, 225-237.
Perkins, S. J., Smith K. F., Williams, S. C., Haris, P. I., Chapman, D. & Sim, R. B. (1994), The secondary structure of the von Willebrand Factor Type A Domain in Factor B of Human Complement by Fourier Transform Infrared Spectroscopy, Journal of Molecular Biology, 238, 104-119.
Edwards, Y. J. K. & Perkins, S. J., (1995) The protein fold of the von Willebrand factor type A is predicted to be similar to the open twisted beta-sheet flanked by alpha-helices found in human ras-p21, 358, 283-286.
Lupas, A., Koster, A. J., Walz, J. & Baumeister, W. (1994) Predicted secondary structure of the 20S proteasome and model structure of the putative peptide channel, FEBS Letters, 354, 45-49.

A strategy for secondary structure prediction

In practice, I recommend getting as many state-of-the-art prediction approaches as possible and combining this with some human insight to give a consensus prediction for the family. If you then align all of your predictions (including ideas you have based on residue conservation) with your multiple sequence alignment you can get a consensus picture of the structure. For example, here is part of an alignment of a family of proteins I looked at recently:

In this figure, three automated secondary structure predictions (PHD, SOPMA and SSPRED) appear below the alignment of 12 glutamyl tRNA reductase sequences. Positions within the alignment showing a conservation of hydrophobic side-chain character are shown in yellow, and those showing near total conservation of non-hydrophobic residues (often indicative of active sites) are coloured green.



Predictions of accessibility performed by PHD (PHD Acc. Pred.) are also shown (b = buried, e = exposed), as is a prediction I performed by looking for patterns indicative of the three secondary structure types shown above. For example, positions (within the alignment) 38-45 exhibit the classical amphipathic helix pattern of hydrophobic residue conservation, with positions i, i+3, i+4 and i+7 showing a conservation of hydrophobicity, with intervening positions being mostly polar. Positions 13-16 comprise a short stretch of conserved hydrophobic residues, indicative of a beta-strand, similar to the example from CheY protein shown above.

By looking for these patterns I built up a prediction of the secondary structure for most regions of the protein. Note that most methods - automated and manual - agree for many regions of the alignment.

Given the results of several methods of predicting secondary structure, one can build up a consensus picture of the secondary structure, such as that shown at the bottom of the alignment above.

from 'A Guide to Structure Prediction' by Robert B. Russell, Biomolecular Modelling Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, P.O. Box 123, London WC2A 3PX, England).

4.1

EXHIBIT 4.1

Pôle Bio-Informatique Lyonnais

Network Protein Sequence @nalysis

NPS@ is the IBCP contribution to PBIL in Lyon, France

Tuesday, July 17th 2001 : A link between NPS@ and Geno3D is available (see news)

Job SOPMA (ID: 1631238) is running on NPS@ server (started on Thu Nov 22 12:48:10 CET 2001).
Results will be shown below. Please wait and don't go back.

SOPMA result for : Example

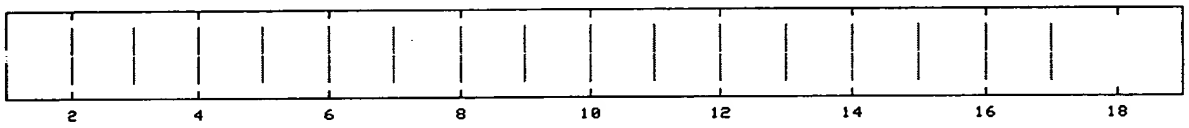
Abstract Geourjon, C. & Deléage, G., SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments., Cabios (1995) 11, 681-684

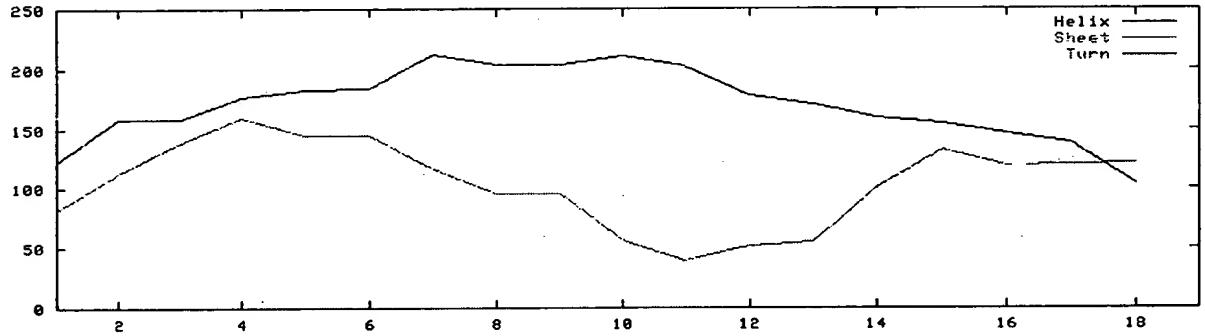
View SOPMA in: [MPSA (Mac, UNIX) , About...] [AnTheProt (PC) , Download...] [HELP]

```
        10
         |
GRYRIYRRIYRRYIRIIIG
cchhhhhhhhhhhhhhhhc

Sequence length :    19

SOPMA :
   Alpha helix      (Hh) :    16 is  84.21%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     0 is   0.00%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :     3 is  15.79%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```






```
Parameters :
   Window width          : 19
   Similarity threshold  :  8
   Number of states      :  3
```

Prediction result file (text): [SOPMA]
Intermediate result file (text): [BLASTP on SWISS-PROT]

4.2 4

EXHIBIT 4.2

Pole Bio-Informatique Lyonnais

Network Protein Sequence @nalysis

NPS@ is the IBCP contribution to PBIL in Lyon, France

Tuesday, July 17th 2001 : A link between NPS@ and Geno3D is available (see news)

Job SOPMA (ID: 1623426) is running on NPS@ server (started on Thu Nov 22 12:55:44 CET 2001).
Results will be shown below. Please wait and don't go back.

SOPMA result for : BP2xxx0

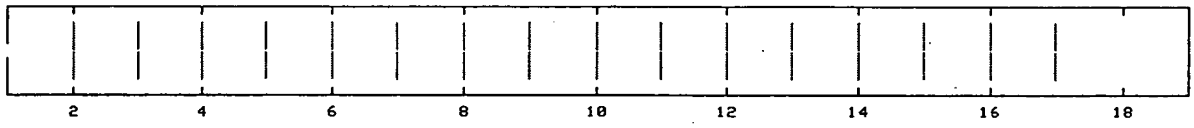
Abstract Geourjon, C. & Deléage, G., SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments., Cabios (1995) 11, 681-684

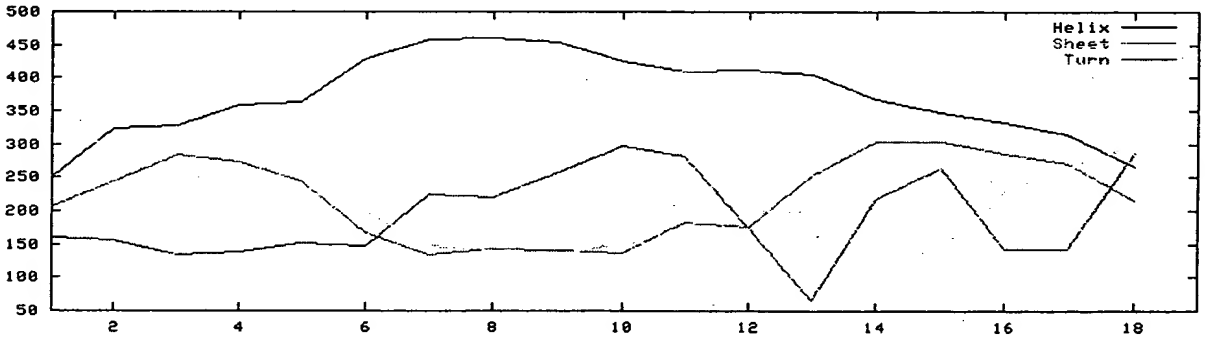
View SOPMA in: [MPSA (Mac, UNIX) , About...] [AnTheProt (PC) , Download...] [HELP]

```
        10
         |
GKWKLFKKAFKKFLKILAC
:--hhhhhhhhhhhhhhhh:

Sequence length :    19

SOPMA :
   Alpha helix      (Hh) :    16 is  84.21%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     0 is   0.00%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :     3 is  15.79%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```






```
Parameters :
   Window width          :  19
   Similarity threshold  :   8
   Number of states      :   4
```

Prediction result file (text): [SOPMA]
Intermediate result file (text): [BLASTP on SWISS-PROT]

User : public@145.117.32.201. Last modification time : Thu Nov 22 12:55:49 2001. Current time : Thu Nov 22 12:55:49 2001 This service is supported by Ministere de la recherche (ACC-SV13), CNRS (IMABIO, COMI, GENOME) and Région Rhône-Alpes (Programme EMERGENCE). Comments .

EXHIBIT 5.1
5.1

Pôle Bio-Informatique Lyonnais
Network Protein Sequence @nalysis
NPS@ is the IBCP contribution to PBIL in Lyon, France

Tuesday, July 17th 2001 : A link between NPS@ and Geno3D is available (see news)

Job HNN (ID: 1630237) is running on NPS@ server (started on Thu Nov 22 12:50:27 CET 2001).
Results will be shown below. Please wait and don't go back.

Hierarchical Neural Network result for : Example

Abstract Guermeur, Y. PhD Thesis

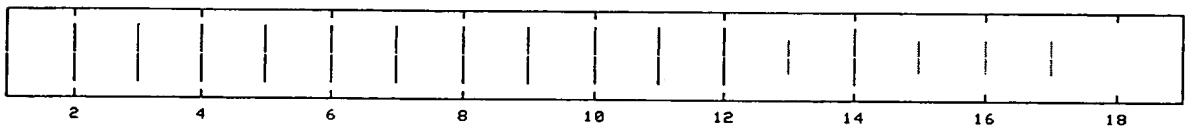
View HNN in: [MPSA (Mac, UNIX) , About...] [AnTheProt (PC) , Download...] [HELP]

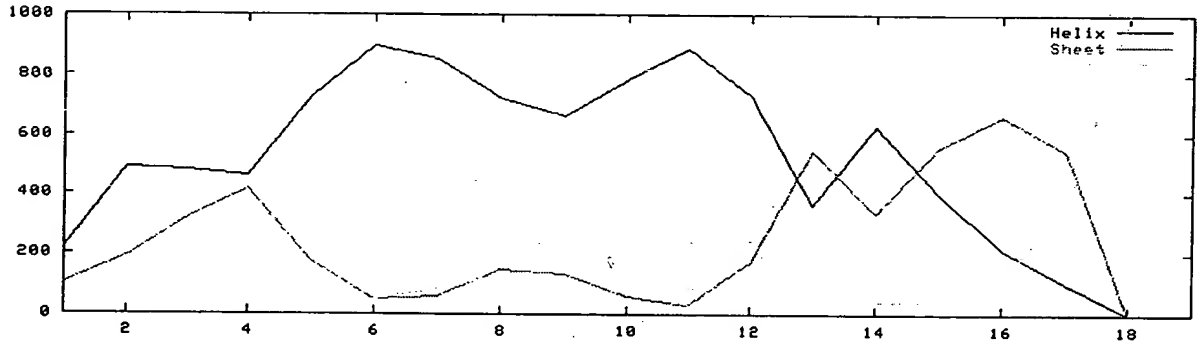
```
        10
         |
GRYRIYRRIYRRYIRIIG
cchhhhhhhhhheheeee
```

Sequence length : 19

HNN :

Alpha helix	(Hh) :	12 is	63.16%
3_{10} helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	4 is	21.05%
Beta turn	(Tt) :	0 is	0.00%
Bend region	(Ss) :	0 is	0.00%
Random coil	(Cc) :	3 is	15.79%
Ambigous states	(?) :	0 is	0.00%
Other states	:	0 is	0.00%





Prediction result file (text): [HNN]

Tuesday, July 17th 2001 : A link between NPS@ and Geno3D is available (see news)

Job HNN (ID: 1632274) is running on NPS@ server (started on Thu Nov 22 12:28:57 CET 2001).
Results will be shown below. Please wait and don't go back.

Hierarchical Neural Network result for : BP2xxx0

Abstract Guermeur, Y. PhD Thesis

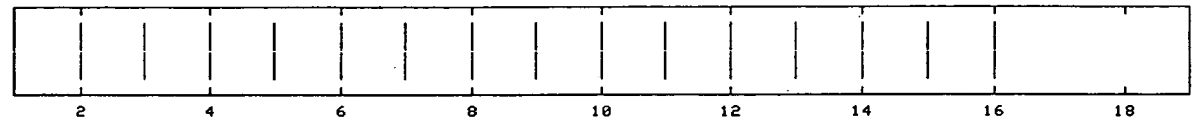
View HNN in: [MPSA (Mac, UNIX) , About...] [AnTheProt (PC) , Download...] [HELP]

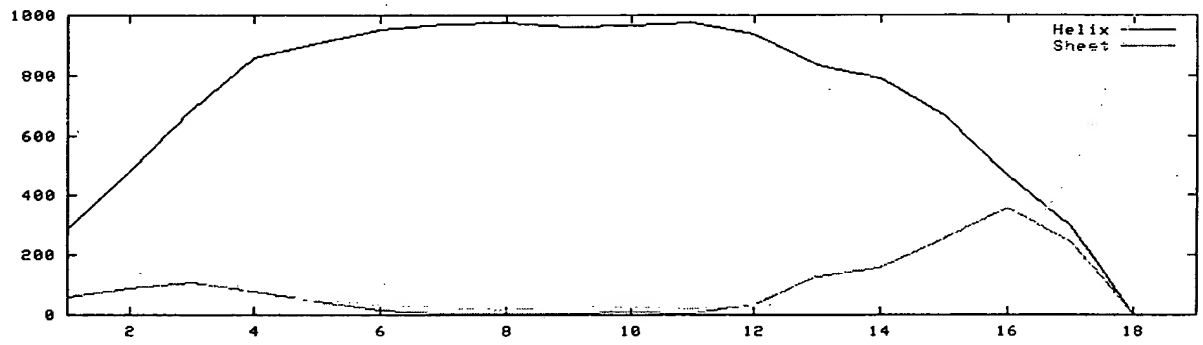
```
        10
         |
GKWKLFKKAFKKFLKILAC
c  hhhhhhhhhhhhhhhh
```

Sequence length : 19

HNN :

Alpha helix	(Hh) :	15 is	78.95%
3_{10} helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	0 is	0.00%
Beta turn	(Tt) :	0 is	0.00%
Bend region	(Ss) :	0 is	0.00%
Random coil	(C) :	4 is	21.05%
Ambigous states	(?) :	0 is	0.00%
Other states	:	0 is	0.00%





Prediction result file (text): [HNN]

User : public@145.117.32.201. Last modification time : Thu Nov 22 12:29:07 2001. Current time : Thu Nov 22 12:29:07 2001 This service is supported by Ministere de la recherche (ACC-SV13), CNRS (IMABIO, COMI, GENOME) and Région Rhône-Alpes (Programme EMERGENCE). Comments .

Exhibit 6

PSIPRED

The protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/)

Liam J. McGuffin, Kevin Bryson and David T. Jones
UCL Bioinformatics Unit, Department of Computer Science,
University College London, Gower Street, London WC1E 6BT, UK

Introduction

The PSIPRED protein structure prediction server incorporates two new methods (PSIPRED and GenTHREADER) and one established method (MEMSAT 2) for predicting structural information about any given protein from its amino acid sequence alone. PSIPRED carries out a reliable secondary structure prediction on a protein,

Predict Secondary Structure (PSIPRED)

PSIPRED is a simple and reliable secondary structure prediction method, incorporating two feed-forward neural networks which perform an analysis on output obtained from PSI-BLAST (Position Specific Iterated - BLAST) (Altschul et al., 1997).

Version 2.0 of PSIPRED includes a new algorithm which averages the output from up to 4 separate neural networks in the prediction process to further increase prediction accuracy.

Using a very stringent cross validation method to evaluate the method's performance, PSIPRED 2.0 is capable of achieving an average Q3 score of nearly 78%. Predictions produced by PSIPRED were also submitted to the CASP4 server and assessed during the CASP4 meeting, which took place in December 2000 at Asilomar. PSIPRED 2.0 achieved an average Q3 score of 80.6% across all 40 submitted target domains with no obvious sequence similarity to structures present in PDB, which placed PSIPRED in first place out of 20 evaluated methods (an earlier version of PSIPRED was also ranked first in CASP3 held in 1998).

It is important to realise, however, that due to the small sample sizes, the results from CASP are not statistically significant, although they do give a rough guide as to the current "state of the art". For a more reliable evaluation, the EVA web site at Columbia University provides a continuous evaluation of the most widely used secondary structure prediction methods.

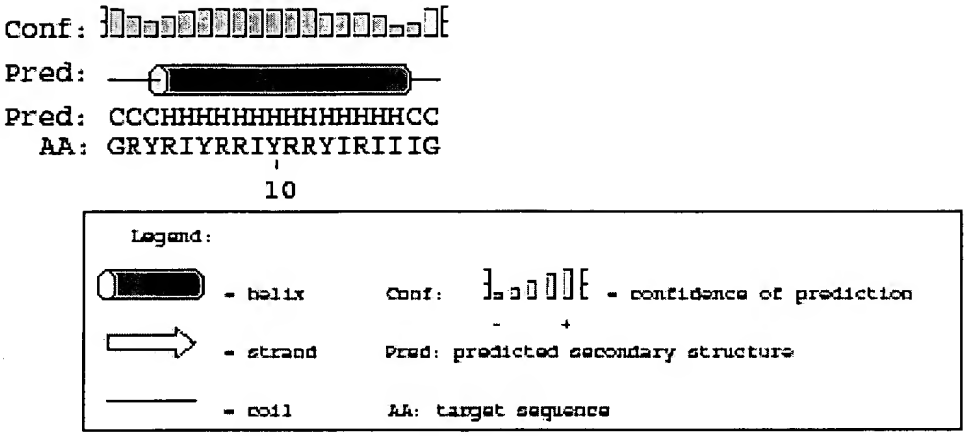
References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman., D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Presented below are the graphical outputs of the PSIPRED secondary structure prediction for the peptide suggested by the Examiner (EP) together with the BP2 peptide:

Exhibit 6 (cont.)

EP



BP2

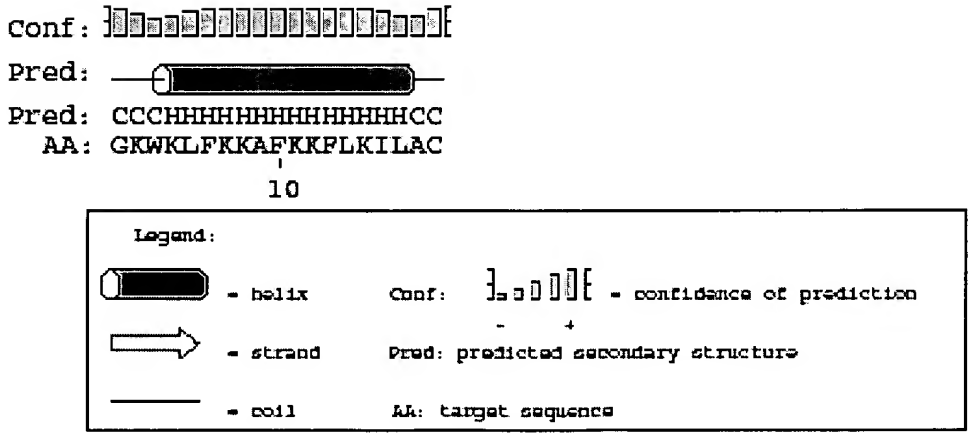
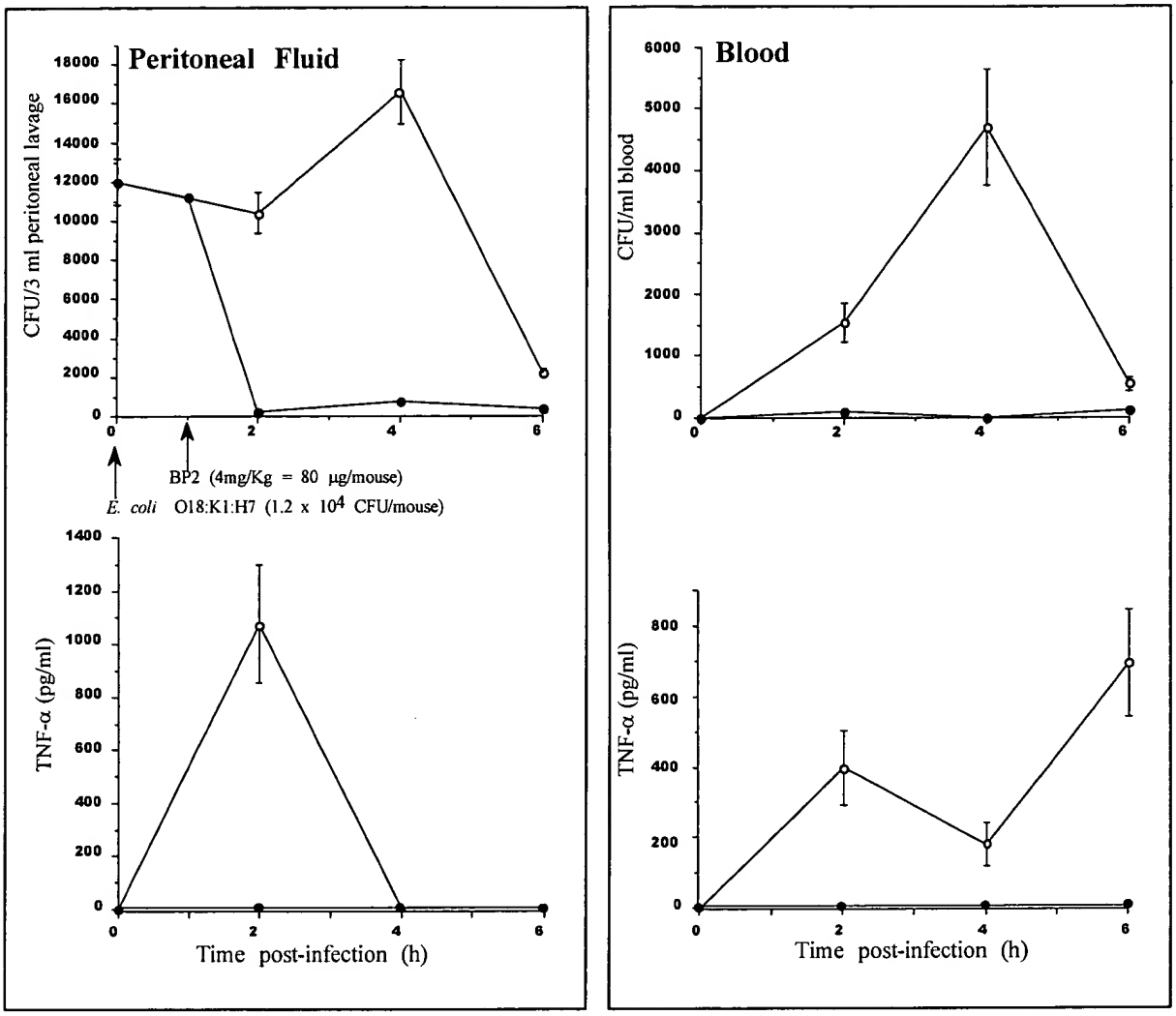


Exhibit 7

Antibacterial and Anti-inflammatory Activity in Experimental Animals



Effect of BP2 treatment on bacterial counts and TNF-α levels in a murine model of lethal Gram-negative peritonitis. Groups of mice were challenged by intraperitoneal administration of *E. coli* 018:K1:H7 Bort (1.2 x 10^4 CFU/mouse). Treatment (closed circles) with BP2 (4mg/Kg) was initiated 1 h post-infection by intraperitoneal administration at a separate abdominal site. Control groups (open circles) only received saline vehicle. Groups of 6 animals per time-point were sacrificed at t = 0, 2, 4 and 6 h and bacterial counts and TNF-α levels determined in peritoneal lavage and blood samples as indicated. Each point represents the mean ± SEM.

Exhibit 8

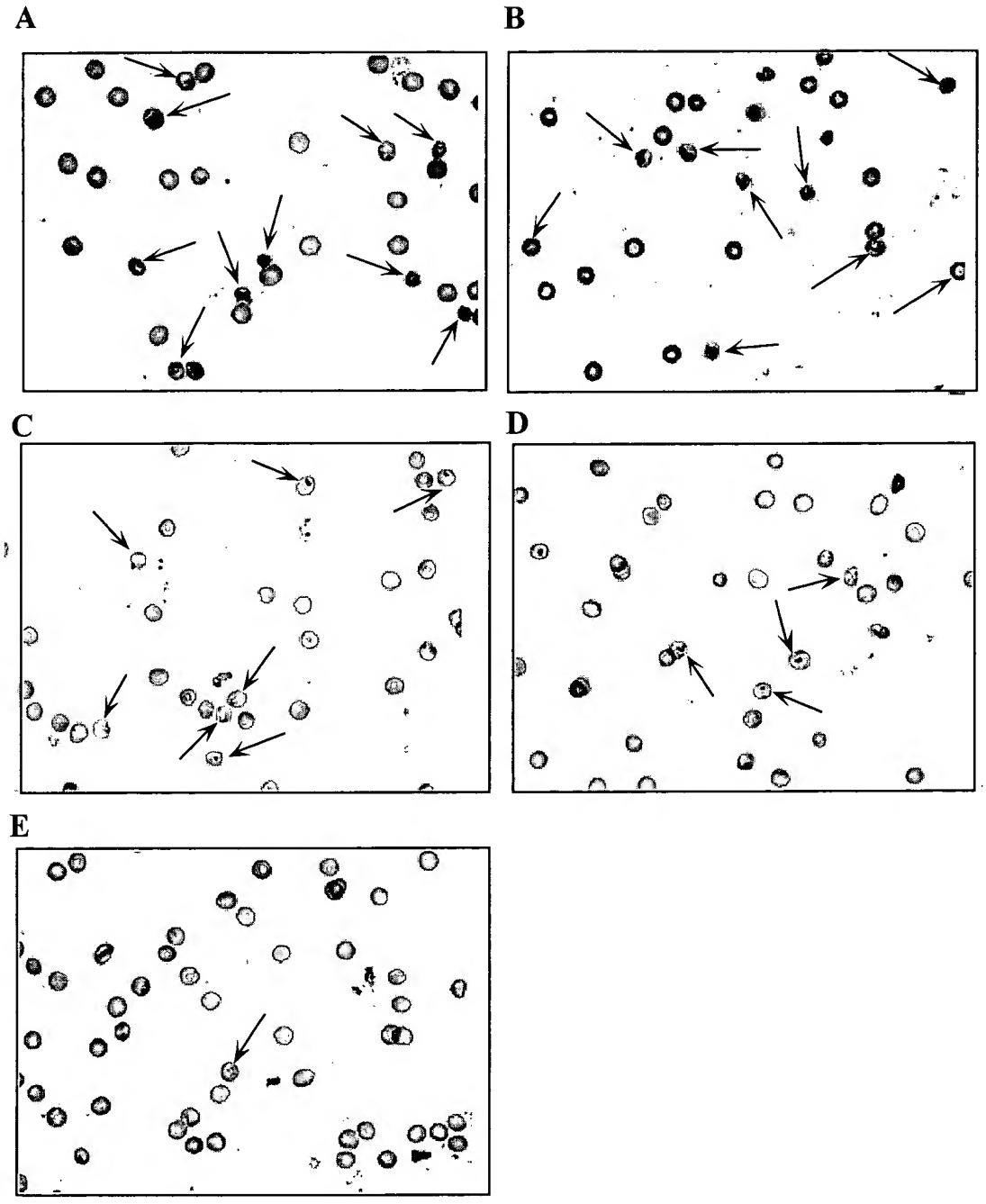
Antiviral Activity

BP1 (μM)	% Reduction of HIV infected PBMC
1	50
5	75

Effect of synthetic peptides on HIV-1 replication *in vitro*. Cultures containing virus-infected human primary peripheral blood mononulear cells (PBMC) were treated by addition of BP1 peptide to final concentrations of 1 and 2 μM . Viral replication was measured after incubation for 24 h at 37 °C by ELISA for CA-p24 capsid protein.

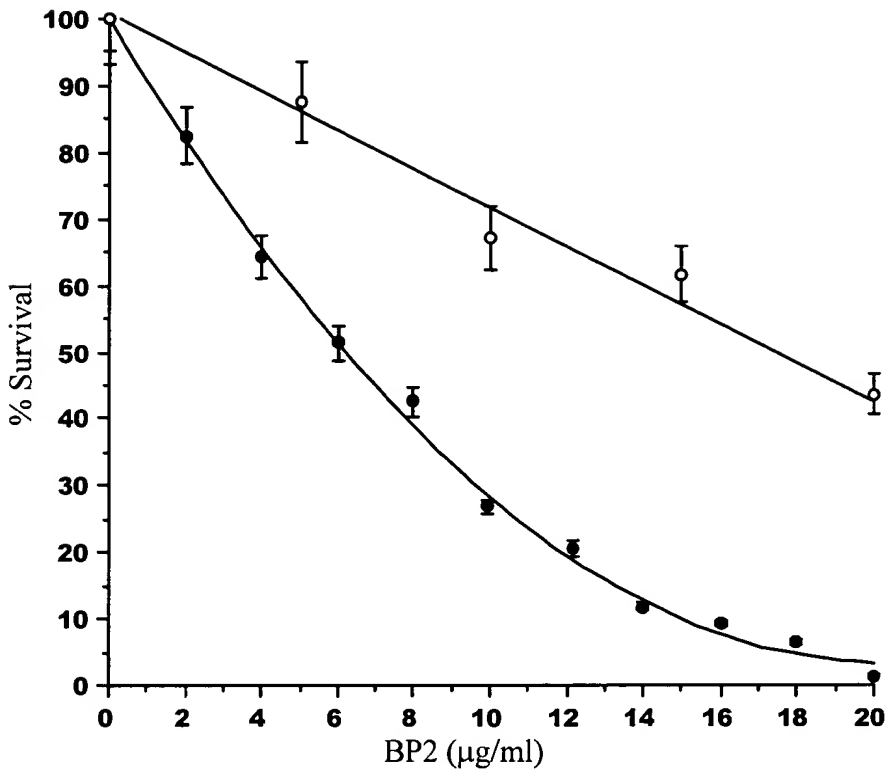
Exhibit 9

Antiparasitic Activity



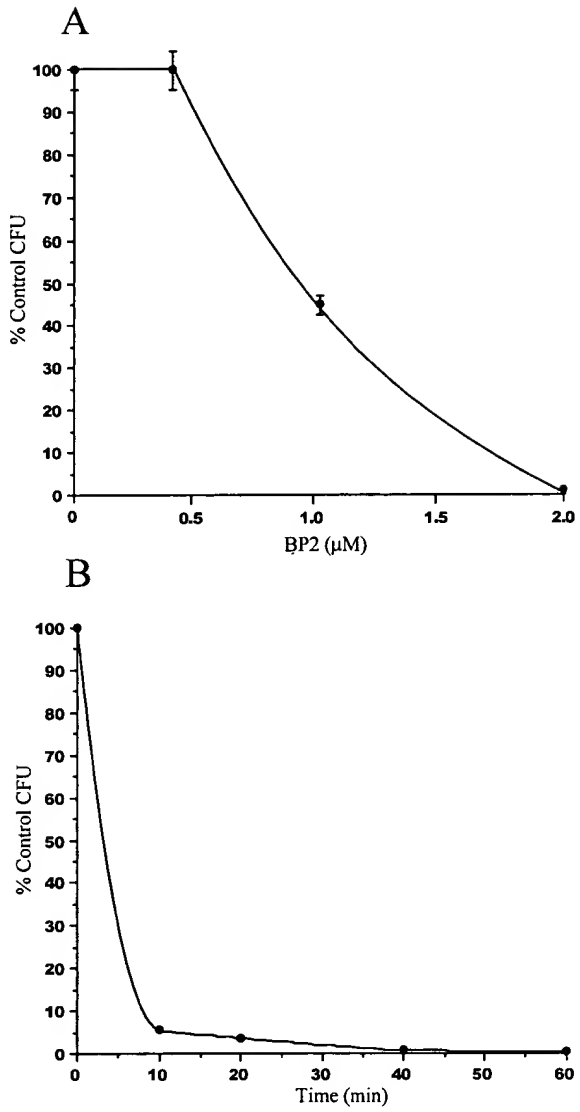
Effect of BP2 on *Plasmodium falciparum* infected human erythrocytes. Cultures containing 4 x 10^4 erythrocytes/ml (Blood Type O^+) and 10% parasite infection were treated by addition of BP2 peptide to final concentrations of 2.5, 5, 10 and 20 μg/ml (Panels **B** to **E**, repectively). The degree of infection relative to the control culture (Panel **A**) was determined visually by histological staining after 24 h at 37°C. Arrows indicate infected erythrocytes in each panel.

Exhibit 10



Effect of BP2 on survival of transformed cell lines. Peptide was added to murine P388 myeloma cells (2.5 x 10^5 cells/ml), indicated by closed circles, or L929 fibroblast cells (10^6 cells/ml), indicated by open circles, in RPMI 1640 medium to the final concentrations indicated. Cell viability was determined after growth for 24 h at 37 °C and 5% CO_2 using a colorimetric MTT cell-viability assay. Survival is expressed as a percantage of untreated controls. The results represent the combined averages of two independent determinations.

Exhibit 11



Effect of BP2 on the survival of serum-resistant Gram-negative bacteria in human whole blood *(ex vivo)*. Blood was collected by venipuncture from healthy human volunteers after informed consent into sterile pyrogen-free tubes containing sodium citrate as anticoagulant (Becton-Dickinson, Lincoln Park, NJ) and immediately used in the experiments. For dose-response analysis (Panel **A**), 5 μl aliquots of peptide dilutions in saline for 0.5 to 2 μM (1-5 μg/ml) were added to 250 μl of whole blood in polypropylene microtubes and incubated for 20 min at 37°C. Control incubations contained only 5 μl of saline vehicle. Subsequently, 20 μl suspensions of early-log phase *E. coli* O18:K1:H7 Bort (10^6 CFU/ml) were added the tubes incubated for 1 h at 37°C. Appropriate dilutions of samples were plated on blood-agar plates and the number of CFU's determined after overnight incubation at 37°C. Time-course analyses (Panel **B**) contained peptide at 2 μM (5 μg/ml) or saline vehicle. Aliquots were withdrawn for plating at selected time-points from 10 min to 1 h. The bactericidal efficacy of the BP2 peptide was expressed as percentage of the control incubations without added peptide. Each value is the mean of replicate determinations in three independent experiments.

Cationic peptides: effectors in innate immunity and novel antimicrobials

Robert E W Hancock

Cationic antimicrobial peptides are produced by all organisms, from plants and insects to human beings, as a major part of their immediately effective, non-specific defences against infections. With the increasing development of antibiotic resistance among key bacterial pathogens, there is an urgent need to discover novel classes of antibiotics. Therefore, cationic peptides are being developed through clinical trials as anti-infective agents. In addition to their ability to kill microbes, these peptides seem to have effector functions in innate immunity and can upregulate the expression of multiple genes in eukaryotic cells. One such function might involve the dampening of signalling by bacterial molecules such as lipopolysaccharide and lipoteichoic acid.
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Since their introduction into human medicine, antibiotics have had an enormous impact on treatment of infectious diseases and the success of invasive medical procedures, such as surgery and chemotherapy. However, the rise in antibiotic resistance threatens to reverse some of these gains.[1] One reason for this development is the paucity of truly novel antibiotics since the introduction of quinolones in the early 1960s. Indeed, for more than 30 years until the release of the streptogramins, synercid, and the oxazolidinone linezolid during the past 18 months, there were no new antibiotic chemical structures. Unfortunately, these antibiotics are niche drugs developed for antibiotic-resistant Gram-positive pathogens, and their restricted activity ranges and toxicity concerns somewhat limit their impact. Thus, it is important to consider new classes of antibiotics. One source is "nature's antibiotics,"[2] the cationic peptides. In this review, I discuss the role of these peptides in innate immunity, and their use as templates in development of a new class of antibiotics that are in phase III clinical trials of topical therapy.

About 20 years ago, the lymph of insects, the granules of human neutrophils, and the skin of frogs were shown to contain peptides that could kill bacteria in culture. Since then, more than 600 cationic peptides have been observed in virtually all species, including bacteria, fungi, insects, tunicates, amphibians, crustaceans, birds, fish, mammals, and human beings.[3] They have generally been referred to as cationic antimicrobial peptides, but in addition to their ability to kill microorganisms directly,[4,5] these substances seem to be able to recruit and promote other elements of host immunity, particularly innate immunity.[6–9] The term cationic amphiphilic peptides, abbreviated to cationic peptides, is therefore used here.

Nature and distribution

Cationic peptides have an enormous variety of sequences and structures,[3] but certain features are common.[2,3,5] The natural cationic peptides are generally 12–50 aminoacids in length, have a net positive charge due an excess of basic lysine and arginine residues over acidic residues, and contain around 50% hydrophobic aminoacids. They fold, owing to the presence of disulphide bridges or contact with membranes, into three-dimensional amphiphilic structures in which the positively charged and hydrophilic domain(s) are well separated from the hydrophobic domain(s). Such a molecule is well suited to interacting with membranes, especially bacterial membranes with their negatively charged and hydrophilic head groups and hydrophobic cores. Nevertheless, both the secondary structures of the cationic peptides, which fit into four classes, and their aminoacid sequences, even within a given class of secondary structures, are quite heterogeneous. The four structural classes include β-sheet molecules stabilised by two or three disulphide bonds, amphipathic α-helices, extended molecules, and loops due to a single disulphide bond (figure 1; the last three classes form upon membrane interaction). The β-sheet and α-helical molecules are by far the most common in nature. Table 1 describes a few representative molecules from nature and related synthetic molecules.

Antimicrobial activities

In the past, with very few exceptions, antibiotics did not have activity against fungi and antifungal drugs did not act against bacteria. However, cationic peptides have a startling range of antimicrobial activities that can include action against most Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites (table 2). Table 1 presents the minimum inhibitory concentrations (MIC) of representative peptides for a Gram-negative bacterium (*Escherichia coli*), a Gram-positive bacterium (*Staphylococcus aureus*), and a fungal pathogen (*Candida albicans*). Various methods are routinely used to assess the ability of peptides to kill bacteria. However, the gold standard is becoming the National Committee for Clinical Laboratory Standards broth dilution method,[11] modified slightly to avoid binding of peptides to plastic surfaces. Generally, the best cationic

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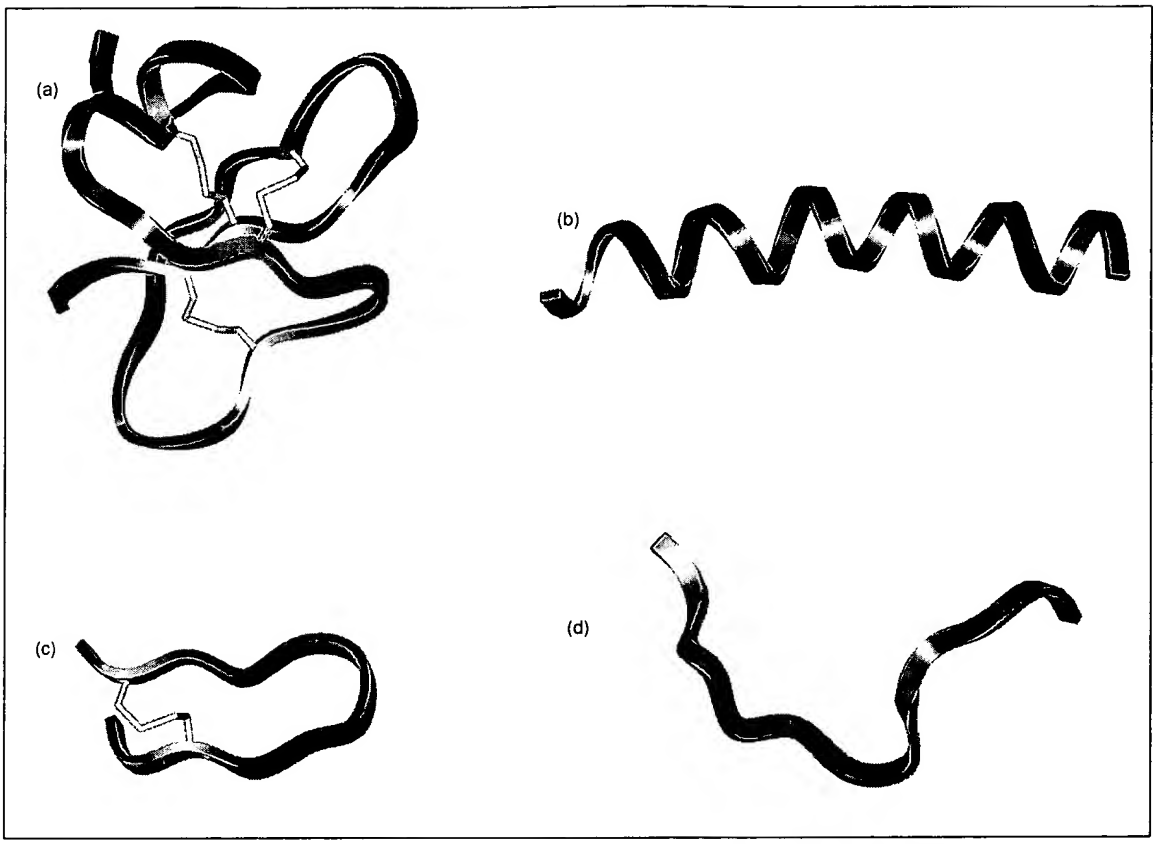

Figure 1. Molecular models of the different structural classes of cationic peptides. These models (taken from the NMR structural database) are based on two-dimensional nuclear magnetic resonance spectroscopy of the peptides in aqueous solution for human β-defensin-2 (HBD-2) or a membrane mimetic condition (other peptides). (a) HBD-2 (PDB code 1FQQ), which forms a triple-stranded β-sheet structure (containing a small α-helical segment at the N-terminus) stabilised by three cysteine disulphide bridges. (b) The amphipathic α-helical structure of magainin 2 (PDB code 2MAG). (c) The β-turn loop structure of bovine bactenecin (model based on the published structure[19]). (d) The extended boat-shaped structure of bovine indolicidin (PDB code 1G89). The backbone structures are shown with the charged regions in blue and the hydrophobic residues in green.

peptides have good activities against most bacteria, and excellent activities (MIC of 1–4 μg/mL) against highly resistant bacteria such as multidrug-resistant *Pseudomonas aeruginosa*, meticillin-resistant *S aureus*, and *Stenotrophomonas maltophilia*.[15,17,18] The cationic peptides are not affected by antibiotic-resistance mechanisms that are limiting the use of other antibiotics; for example, they are as active against meticillin-resistant *S aureus* as they are against meticillin-sensitive strains. A few antibiotic-resistance mechanisms that affect antimicrobial peptides have been described,[19–21] but most seem to have only a moderate (two to four fold) effect on MIC. Indeed, selection of resistant mutants against most peptides is quite difficult, with more than 12 passages on 50% MIC of antibiotic being required to increase resistance by two fold. However, there are a few resistant bacterial species, including *Burkholderia cepacia* (by virtue of its unique outer membrane[14]) and *Serratia* spp.

Cationic peptides are bactericidal, with the MIC and minimum bactericidal concentration coinciding or differing by no more than two fold. They kill bacteria very rapidly (figure 2) compared with conventional bactericidal antibiotics.[15,17]

A large number of, but by no means all, cationic peptides have useful antifungal activities. Indeed, given the importance of fungal diseases of plants, it is perhaps not surprising that many plant peptides are selective for fungi,[5] as are certain insect peptides such as drosomycin.[6] Few studies have been done to study the antifungal spectrum of cationic peptides, and we know little about the specific mechanism of action, although various processes have been described, including morphological distortions, rapid ion fluxes,[23] and inhibition of energised mitochondria.[24]

Another target that has been even more poorly defined is eukaryotic parasites. Selected peptides have activity against protozoa, including trypanosomes, malaria parasites, and nematodes.[25–27] Activity against cancer cells has also been reported,[28,29] although there is doubt as to whether such peptides have the necessary selectivity for malignant over normal cells, and some peptides can be quite toxic. Some peptides, including defensins, indolicidin, polyphemusin, and melittin, also have activity against viruses including HIV, herpes simplex virus, influenza A virus, and vesicular stomatitis virus.[30] Mechanisms have been reported to include blockage of virus-cell fusion and the activity of HIV long terminal repeats.

Table 1. Sequences and properties of selected natural and synthetic cationic peptides

Peptide	Class*	Derivation	Sequence†	MIC (mg/mL)‡ *Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
HNP1	β3(α)	Human neutrophils	AC$_1$YC$_2$**R**IPAC$_3$IAGE**RR**YGTC$_3$IYQGRLWAFC$_2$C$_1$	>50	3·1§	
HBD-3	β3(β)	Human skin	GIINTLQ**K**YYC$_1$**R**VRGG**R**C$_2$AVLSC$_3$LPKEEQIG**K**C$_2$ STR**GRK**C$_1$C$_3$**RRKK**	~6‖	~12	~6
Polyphemusin I	β2	Horseshoe crab	**RR**WC$_1$F**R**VC$_2$Y**R**GFC$_2$Y**RK**C$_1$**R**-NH$_2$	0·13	0·5	1
Protegrin	β2	Pig	**RGGR**LC$_1$YC$_2$**RRR**FC$_2$VC$_1$VG**R**-NH$_2$	0·5	2	4
IB-367	β2	Synthetic	**R**GGLC$_1$YC$_2$**R**G**R**FC$_1$VC$_2$VG**R**-NH$_2$	1	4	32
Magainin II	α	Frog	GIG**K**FLHSA**KK**FG**K**AFVGEIMNS	50	>100	
MSI-78	α	Synthetic	GIG**K**FL**KK**A**KK**FG**K**AFVKIL**KK**-NH2	2	16	
Cecropin B	α	Silk moth	**K**WKVF**KK**IEK**M**GR**N**I**R**NGIV**K**AGPAIAVLGEA**K**AL-NH$_2$	5	>200	
CP-α2	α	Synthetic	**K**WK**K**FI**KK**IGIGAVLK**V**LTTGLPAL**K**LT**KK**	2	16	64
Indolicidin	E	Bovine neutrophils	ILPW**K**WPWWPW**RR**-NH$_2$	16	8	4
CP-11CN	E	Synthetic	IL**KK**WPWWPW**RRK**-NH$_2$	4	16	16
CP-10A	α	Synthetic	ILAW**K**WAWWAW**RR**-NH$_2$	8	4	16
Bactenecin	C	Bovine neutrophils	**R**LC$_1$**R**IVVIRVC$_1$**R**	8	32	64
BacW2R	C	Synthetic	**RR**LC$_1$**R**IVWVI**R**VC$_1$**R**	2	2	>64
Gramicidin S	βC	Bacteria	Cyclic (**LO**VPF^d**LO**VPF^d)	8	2	2
Polymyxin B	CL	Bacteria	Isooctanoyl **BTBB**(**BF**^{d}**LBBT**) cyclised	0·5	32	32

*Classes are: β, beta-structured (number refers to the number of disulphide bridges; α or β after the number refers to the family of mammalian α or β defensins from which the peptides come); α, amphipathic α-helical; E, extended structure; C, cyclic; L (polymyxin only), lipopeptide. †One-letter aminoacid code with the following additions. Residues positively charged at neutral pH are in bold. Parentheses indicate aminoacids that are cyclised. Superscript d represents the D-enantiomer; all other aminoacids are L-form. The subscript numbers represent aminoacids that are joined by cysteine disulphides. O, ornithine; B, diaminobutyrate; ×, 2,3-didehydrobutyrine; U, 2,3-didehydroalanine; Z, a-aminobutyrate. ‡MICs were generally determined by modified NCCLS broth dilution assays,[11] and results from the our laboratory are generally used for consistency. Inhibitory concentrations are greatly affected by the method used and the salt content of the assay medium. By the radial diffusion assay method of Lehrer and colleagues, killing at much lower concentrations can be demonstrated. §MICs were not done for *S aureus* but were done for another Gram-positive bacterium *Enterobacter cloacae*,[12] and I assumed here that they are similar. ‖Concentrations resulting in 100% killing.[13]

Peptides are generally found at quite low concentrations in the normal tissues of mammals, and several different peptides can be found in a single tissue.[8] Indeed, their natural role may involve synergy both with each other and with other agents in the host. In frogs, magainin 2 shows synergistic killing with the peptide PGLa,[31] and this finding has been extended by checkerboard titration studies with various cationic peptides.[12] Synergy has also been shown with lysozyme,[12] with various antibiotics against selected wild-type and mutant bacteria,[14] and with antifungal agents, antiprotozoal agents, and the anticancer drug doxorubicin against fungi, protozoa, and cancer cells, respectively.

Mechanism of antibacterial action

An enormous amount of work has been invested in model membrane studies.[32–35] However, although the findings of such studies are consistent with the central observation that the interaction of cationic peptides with the bacterial cytoplasmic membrane is an essential step in the peptides' bactericidal activity, they have also led to a broad variety of hypotheses to explain bacterial killing. Gram-negative bacteria have an additional outer membrane barrier to cross, and the self-promoted uptake hypothesis[36] appears to describe how such uptake occurs (figure 3). According to this hypothesis, the cationic peptides interact with the highly negatively charged surface of the outer membrane and displace magnesium ions that normally partly neutralise this charge. The high negative charge is carried by the anionic glycolipid lipopolysaccharide, which fills the outer monolayer of the outer membrane. The cationic peptide then distorts the outer membrane either by strongly binding to the lipopolysaccharide or by neutralising charge over a patch of the outer membrane. The peptide is then proposed to insert into and translocate across this bilayer. Since many cationic peptides are selective for Gram-negative over

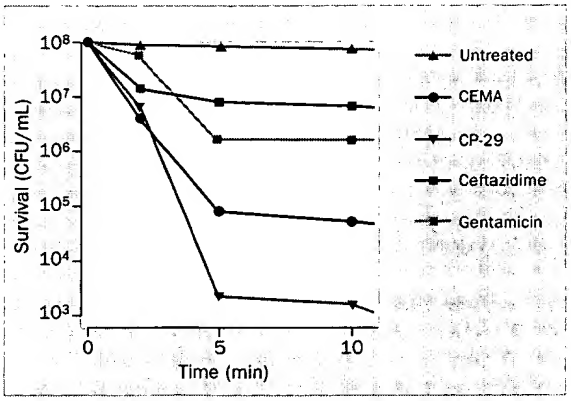

Figure 2. Rate of killing of E coli *UB1005 in Mueller-Hinton broth by four fold the MIC of cationic peptides compared with conventional antibiotics Adapted from published data.[22] Ceftazidime at 2 μg/mL, gentamicin at 0·5 μg/mL, CEMA at 4 μg/mL, and CP-29 at 2 μg/mL.*

Table 2. Activities of cationic antimicrobial peptides and some examples of peptides with those activities

Activities of antimicrobial peptides	Example peptides*
Broad-spectrum antibacterial	Protegrin, IB-367, MSI-78, indolicidin, CEMA, gramicidin S, polyphemusin,
Anti Gram-negative bacteria	Polymyxin B
Anti Gram-positive bacteria	HNP1
Synergy with conventional antibiotics	CEMA, magainin II, MSI-78, IB-367
Antifungal	Protegrin, CEMA, indolicidin, gramicidin S, polyphemusin
Synergy with conventional antifungals	Indolicidin
Antiviral (HIV, HSV)	Indolicidin, polyphemusin, protegrin
Anticancer	CEMA, indolicidin
Synergy with conventional anticancer agents	Indolicidin
Antiparasite	Magainin II, indolicidin
Antiendotoxin	CEMA, polyphemusin variants
Wound healing	Magainins, PR39
Chemotactic	HNP-1

*In addition to the peptides described in table 1, CEMA (previously termed CP28 or MBI-28) is an α-helical peptide.[14] Polyphemusin is a β-hairpin peptide from horseshoe crabs, structurally related to protegrin,[15] and PR39 is an extended peptide.[16]

Gram-positive bacteria, and since their action on the outer membrane causes protrusion of the outer membrane or blebs at discrete points on the cell surface (figure 4), we can assume that this interaction with the outer membrane focuses the peptide to attack discrete areas of the cytoplasmic membrane. Also, the distortion of the outer membrane appears to provide a partial explanation for the synergistic activities of antimicrobial peptides described above. However, these outer-membrane interactions do not result in cell death, because peptides that interact well with the outer membrane, but do not kill cells well, have been demonstrated.[37]

Having crossed the outer membrane (or the thick cell wall in the case of Gram-positive bacteria), the peptides approach the cytoplasmic membrane. Model studies have clearly shown, and virtually all researchers agree, that the peptides interact electrostatically with the anionic surface of the bacterial cytoplasmic membrane and this interaction induces insertion of the peptide into a position parallel to the membrane at the interface of the hydrophilic head groups and hydrophobic fatty acyl chains of the membrane phopholipids.[32-35] During insertion, the peptide folds into a membrane-bound structure, if not already folded as a result of disulphide bridging or passage across the outer membrane. After parallel membrane insertion, four outcomes have been proposed on the basis of model membrane studies and to some extent intact cell studies (figure 4). Although several reviewers have suggested lysis as an outcome, there is little evidence for complete dissolution of the majority of bacterial cells at the minimum effective concentration. The left-hand inset of figure 4 shows intact *E coli* treated with 32 times the MIC of the peptide CEMA, without apparent loss of underlying cell shape. There is, however, a striking change in outer-membrane morphology, which adopts a blistered appearance. The right hand panel of figure 4 shows no loss of integrity in *S epidermidis* despite treatment with ten times the MIC of

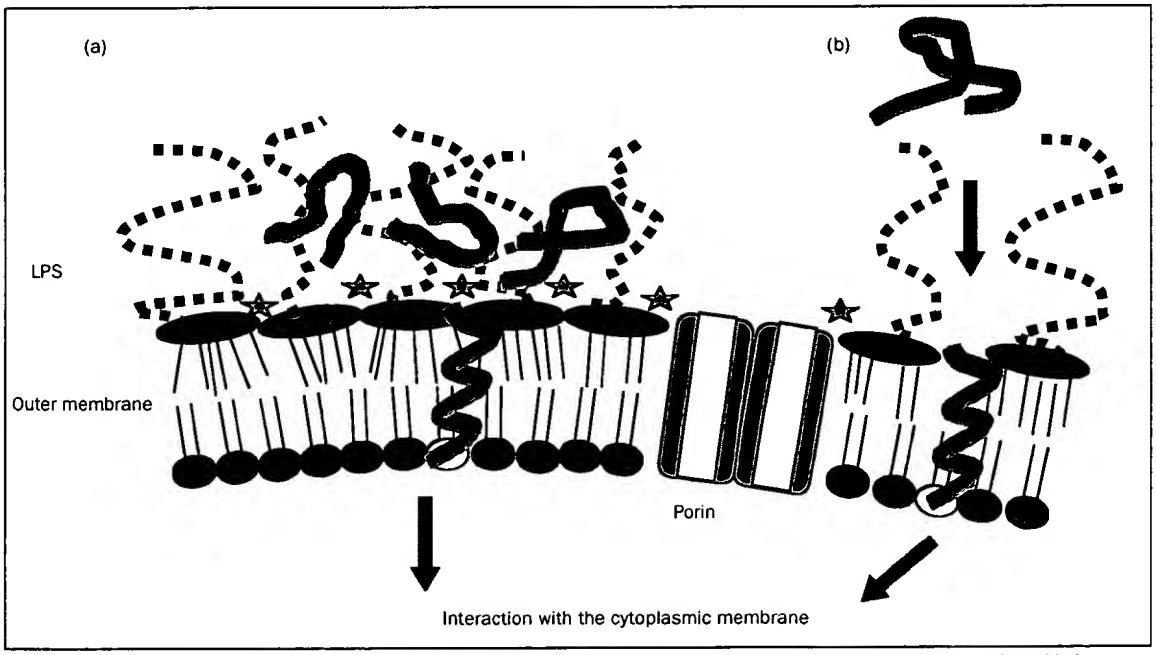

Figure 3. Self-promoted uptake of cationic peptides across the outer membrane. Unfolded cationic peptides are proposed to associate with the negatively charged (mainly due to the presence of highly anionic lipopolysaccharide [LPS]) surface of the outer membrane. They then either neutralise the charge over a patch of outer membrane, creating cracks through which the peptide can cross the outer membrane (a) or actually bind to the divalent cation binding sites on lipopolysaccharide, and disrupt the membrane (b). Once the peptide has crossed the outer membrane, it will interact with the negatively charged surface of the cytoplasmic membrane.

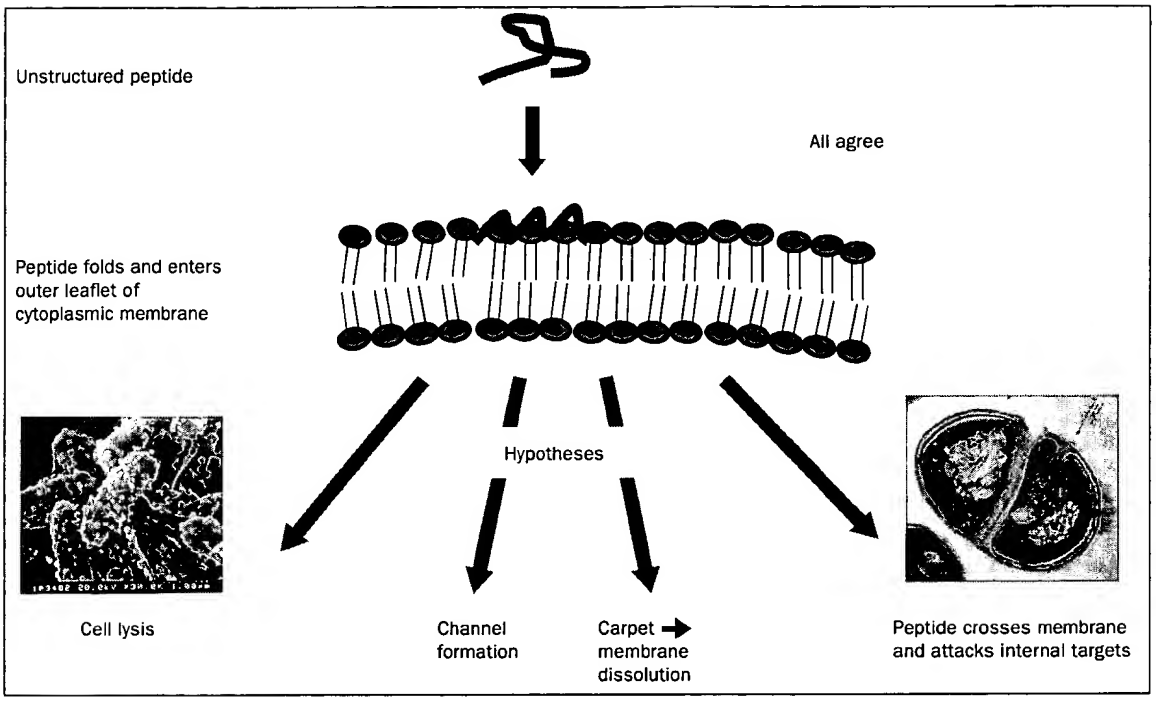

Figure 4. Peptide interaction with the cytoplasmic membrane of bacteria. The mechanism of entry of peptides to cells is undisputed, but there are four hypotheses for how they kill cells. One view is that they lyse cells, but E coli *is not lysed by treatment with 64 μg/mL CEMA for 30 min (inset on left). Another model suggests that when many peptide molecules insert into the membrane interface, they aggregate into a micelle that spans the membrane or flip-flop across the membrane under the influence of the large transmembrane electrical potential gradient;[30,38] inset on right represents the interaction of a linear bactenecin variant Bac2A (with the cysteines changed to alanines) with* Staphylococcus epidermidis. *Some of the events that can be seen are condensation of the DNA indicating uptake of the peptide into the cytoplasm, and cell division defects including an aberrant septum and the initiation of a false septum.*

Bac-2A, and obvious changes to the cell (DNA condensation and a false septum).

Two other hypotheses suggest that the peptides reach a high concentration at the outer surface of the cytoplasmic membrane and reorient to a position perpendicular to the cytoplasmic membrane to form channels with regular structure[33] or cause catastrophic breakdown of cytoplasmic-membrane integrity (the "carpet" model).[35] In these models, the mechanism of action is thought to be breakdown of cytoplasmic-membrane integrity. However, although virtually all cationic amphiphilic peptides cause cytoplasmic-membrane permeabilisation if applied at high enough concentrations, many do not depolarise (break down the membrane potential gradient of) intact cells at concentrations leading to cell killing.[38] Indeed, the toad histone-derived peptide buforin can translocate across lipid bilayers without affecting the membrane barrier function,[39] and all peptides active against Gram-negative organisms are by definition capable of translocation across at least one bilayer, the outer membrane.[32] For this reason, and to explain the results of studies on model membranes and bacterial cytoplasmic-membrane interaction for a wide variety of peptides, we proposed the micellar aggregate channel hypothesis,[32,38] which postulates that the peptides reorient according to concentration and possibly the cytoplasmic-membrane electrical potential gradient (− 140 mV oriented as internal negative) to form micelle-like aggregates that provide informal channels for the movement of ions across the membrane. According to planar bilayer studies,[38] such channels can vary in both size and duration but can last as little time as microseconds; they are proposed to collapse in such a way that the peptide can move to the outer or inner monolayer in a parallel configuration, with the inner monolayer peptide having been translocated. In this model, various targets are possible, including the cytoplasmic-membrane barrier, cell-wall synthesis or degradation, cell division, macromolecular synthesis, or even selective enzyme targets (eg, figure 4, inset on left). Individual peptides might "prefer" a particular target, but

Table 3. Influence of selected cationic antimicrobial peptides given intraperitoneally as a single dose of 8 mg/kg to neutropenic mice infected with *P aeruginosa* and to galactosamine-sensitised mice treated with endotoxin

	Survival (%)	
Peptide*	*P aeruginosa* infection	Endotoxin
None	6	0
CEMA	43	78
CPα2	80	ND
Polyphemusin	20	10
PV5	40	50

*Peptides are the cecropin-melittin hybrid α-helical peptides CEMA and CPa2[14] and horseshoe crab polyphemusin and a variant PV5.[15] ND=not determined.

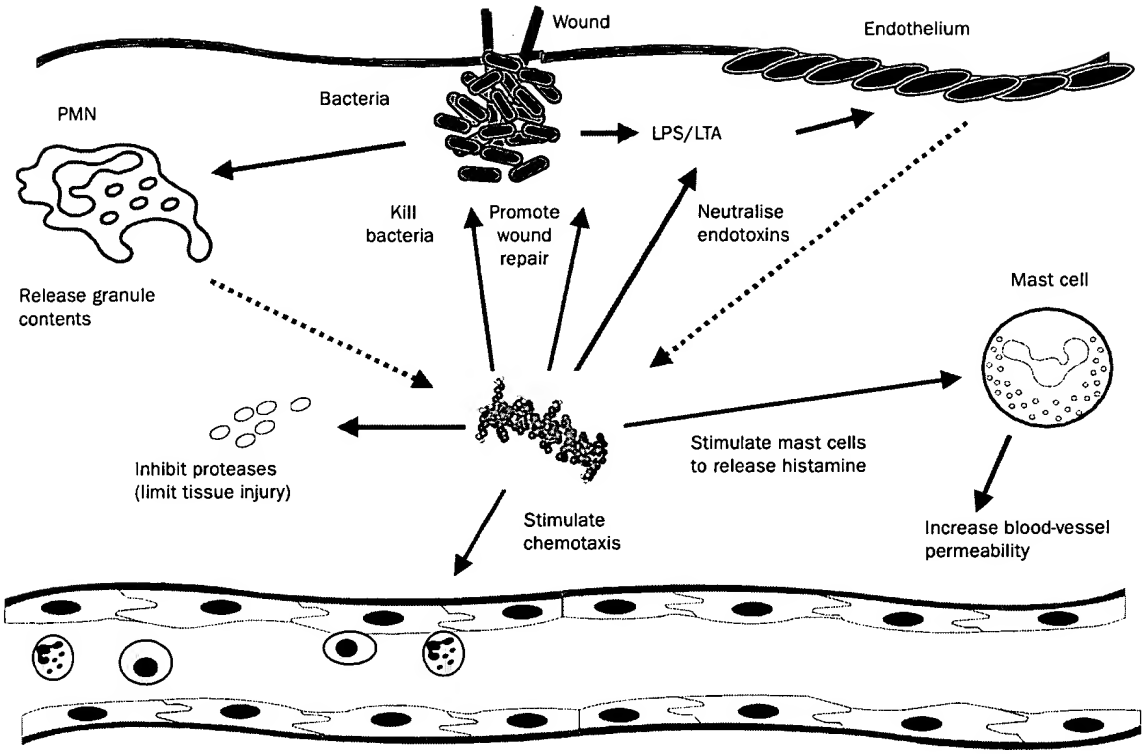

Figure 5. Scheme illustrating the proposed role of cationic peptides in innate immunity with specific reference to events that occur in chronic inflammation. Dotted arrows represent events that lead to increased production of extracellular cationic peptides, solid red lines actions of the peptides, and solid pink lines events due to the bacteria. The overall scheme presented is a mosaic of the separate effects.[7,8] LPS=lipopolysaccharide; LTA= lipoteichoic acid; PMN=polymorphonuclear leucocytes.

the existence of secondary targets and the proposed dependence of these targets on physical interactions (charge–charge and hydrophobic interactions) might help to explain why development of resistance against cationic peptides is difficult. The net effect is that some monomers will be translocated into the cytoplasm[39] and can dissociate from the membrane and bind to cellular polyanions such as nucleic acids.[22]

Role in innate immunity

There is much evidence that cationic peptides have an important role in living hosts.[6–9,40] Since a single host can contain up to about 35 different antimicrobial peptides from all structural classes,[3] elimination of all of these peptides at once is not possible, so clever manipulations must be made to assess in-vivo importance. For example, in drosophila, mutations in regulatory or signalling genes can affect the expression of many peptides and increase susceptibility to bacterial or fungal infections.[41] In mice, Wilson and colleagues[42] identified the enzyme matrilysin, which brings about processing of intestinal preprodefensins to mature defensins. Knocking out the matrilysin gene denuded the small intestine of mature defensins and increased susceptibility to infection with ingested organisms by ten fold. Cole and colleagues[43] similarly applied protease inhibitors specific for the protegrin-processing enzyme to wounds on the skin of pigs, decreasing the amounts of active protegrin and the ability to resist a bacterial challenge. Although similar experiments cannot be done in healthy human beings, patients with specific granule deficiency syndrome lack α-defensins and have severe and frequent bacterial infections.[5] Another way of assessing activity in vivo is to increase the amount of cationic peptides. This has been done in two ways. Bals and co-workers[44] introduced an adenovirus carrying the transgene for the human peptide LL-37 into the lungs of mice, leading to protection against *E coli* infections and endotoxin. Many other studies have shown that exogenously introduced peptides can protect against endotoxaemia and bacterial and fungal infections (table 3).[8,9]

There is no doubt that cationic peptides can be found in high (bactericidal) concentrations at certain sites in the host.[8,9] For example, the concentration of defensins in the azurophilic granules of neutrophils can be as high as 10 mg/mL, whereas various insect peptides when induced can circulate in the lymph at concentrations of up to 100 μg/mL or more. In these cases, we can assume that such peptides function in innate immunity to kill infectious agents directly. On the other hand, certain body sites in human beings contain quite low concentrations of peptides (eg, airway surface fluids contain 0·3 to 8 μg/mL of human β-defensin-2[45,46] and 2 μg/mL of the α-helical cathelicidin LL-37[47]). These concentrations can be increased for some peptides by infection, but except in pathological,

inflammatory diseases, concentrations still seem to be below those needed to kill infectious agents. Thus, the fact that these peptides have various activities that are relevant to innate immunity is of great interest.

In particular, peptides can neutralise host responses to conserved bacterial signalling molecules such as endotoxic lipopolysaccharide from Gram-negative bacteria,[48] lipoteichoic acid from Gram-positive bacteria,[9] and unmethylated CpG DNA from all bacteria (MG Scott, REW Hancock, unpublished). Such molecules interact with Toll-like receptors on the surface of host cells to trigger signalling cascades and cause upregulation of cytokines, such as tumour necrosis factor (TNF) and interleukin 6, chemokines like macrophage inflammatory protein 1α and 1β, and dozens of other gene products.[48] Although low concentrations of these signalling molecules cause beneficial proinflammatory responses and fever, too sustained or vigorous a response can lead to systemic circulation problems, organ failure, and even death.[49] Cationic peptides can neutralise these responses, for example by suppressing the upregulation by lipopolysaccharide of TNF expression both in macrophages in culture and in sensitised mice.[14] This action results in protection against endotoxaemia and death.[50] The mechanism of suppression involves both inhibition of binding of lipopolysaccharide to a serum factor lipopolysaccharide-binding protein, in addition to a proposed direct action on host cells.[50] Microarray experiments showed that the effects of the cationic peptides CEMA[48] and LL-37[50] are selective, in that of the 52 genes observed to be upregulated by lipopolysaccharide, only about 35 were suppressed to differing extents in the presence of cationic peptides. Since the natural bacterial flora of animals can conceivably release small amounts of bacterial signalling molecules, one role of cationic peptides at the surface of cells may thus be to prevent the induction of inflammatory responses by these bacteria.

Cationic peptides also have various interactions that relate to innate immunity, including stimulation of the chemoattraction of monocytes and neutrophils, promotion of histamine release from mast cells, inhibition of tissue proteases, and stimulation of wound healing (figure 5). Microarray experiments have confirmed the ability of cationic peptides to upregulate selectively the expression of more than 30 genes.[48] Furthermore, there is strong circumstantial evidence for involvement of specific receptors in the chemotactic response stimulated by peptides.[47] Nevertheless, to date these results remain fragmentary and there are as yet no data in animal models to confirm that such interactions are important.

Clinical development

The general proof of principle for the use of cationic antimicrobial peptides as therapeutic agents has already been established.[2] Two bacterium-derived, non-ribosomally synthesised cationic peptides, gramicidin S and polymyxin B,[52] have already found use in topical creams and solutions. However, these molecules tend to be toxic and this characteristic limits their potential for systemic use. Interestingly, neutralisation of the amino groups of polymyxin E with methane sulphonate creates a prodrug, colomycin, that can be used systemically.

Cationic peptides have had a chequered history in the clinic and currently only five clinical trials of topical treatment are underway. These include a phase III trial for therapy, by a protegrin-like molecule, IB-367, of oral mucositis, a painful ulcerative polymicrobial infection most commonly associated with radiotherapy or chemotherapy for cancer. There were also phase II clinical trials of IB-367 in aerosol formulation for *P aeruginosa* lung infections in people with cystic fibrosis. An indolicidin, MBI-226, is undergoing phase III clinical trials for sterilisation of insertion sites for central venous catheters; these trials have been fast-tracked by the US Food and Drug Admistration. Other indolicidin-like peptides are being investigated for therapy of acute acne (in phase II clinical trials).

However, until cationic amphiphilic peptides can be used systemically they will not achieve their true potential, and the barriers that must be overcome are discussed below.

Barriers

Any new class of pharmaceuticals faces a series of tests that must be overcome to achieve success in the clinic. In general, these include demonstration of good activity, appropriate formulation, an appropriate manufacturing method, sufficient stability in vivo, and low toxicity. Since there are virtually no published data on many of these topics for cationic peptides, the following discussion is somewhat speculative.

There is no doubt that cationic amphiphilic peptides have excellent antimicrobial activity in vitro and, in principle, represent almost ideal candidate drugs. There is some evidence that this good in-vitro activity can translate to in-vivo activity in animals, but studies in which protection is complete are rare, probably owing to formulation or stability issues. An example is observed for polyphemusin I, a β-hairpin peptide from horseshoe crabs, which in vitro is the most active peptide we have studied to date, but in animal models has no activity against infections.[15] By contrast, modest sequence modifications can create peptides with slightly lower in-vitro activity but reasonable (although incomplete) protection in infections of animals.[15] The most obvious cause of poor or incomplete in-vivo activity is lack of stability due to the action of host proteases. Ways of overcoming this instability might be improved formulation (eg, in liposomes, masking the peptide), use of the prodrug approach as discussed above for colomycin, development of cyclic peptides with strained peptide bonds that are more resistant to proteolysis, and sequence modifications. In the last case,[51] cationic peptide precursors can be protease inhibitors,[52] so moderate changes in sequence might convert a protease substrate to a protease inhibitor.

Search strategy and selection criteria

I have followed the field of antimicrobial peptides closely for the past 15 years with an automated monthly search of *Current Contents* and *Biological Abstracts*, using key words that include general terms such as antimicrobial peptides and the names of specific peptides of note. In addition, manual searches of Medline were carried out, and the press releases of the major companies involved in the field were examined. The amount of literature in this field is becoming voluminous, so the references used were highly selected to present specific points of note, and many general review articles rather than the first paper presenting a given topic. I recommend that readers also examine review articles referenced herein to find many other excellent papers.

Conclusion

Antimicrobial peptides offer an enormous range of useful activities ranging from antimicrobial to immunomodulation. They are proceeding to the clinic as topical antibiotic agents. However, elucidation of their biological importance in innate immunity and realisation of their full clinical potential will require much more effort.

Acknowledgments

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Curriculum vitae

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Appointments and Experience

Senior Scientist (1995-present)
Dept. of Experimental Internal Medicine , Academic Medical Center, University of Amsterdam,
Postdoctoral Scientist (1994-1995)
Dept. of Medical Microbiology, Vrije Universiteit, Amsterdam

Anti-infective Peptide Therapeutics (1994-present)

- Initiated and established a project for the development of novel synthetic antimicrobial peptide therapeutics for the prevention/treatment of septic shock caused by multi-drug resistant bacterial pathogens.
- Generated a new class of Bactericidal Peptides (BP), with antibiotic and anti-inflammatory activity by rational design and molecular modeling.
- Demonstrated potent broad-spectrum bactericidal activity against multi-resistant clinical isolates, high-affinity lipopolysaccharide (LPS) and lipoteichoic acid (LTA) -binding and effective endotoxin-neutralizing activity of a number of candidate peptides in preclinical studies.
- Demonstrated potential therapeutic properties of the lead peptide BP2 in animal models of experimental Gram-negative endotoxemia, peritonitis, pneumonia and Gram-positive bacteremia.
- Prepared an international patent application "Novel synthetic peptides with antimicrobial and endotoxin-neutralizing properties for management of the sepsis syndrome". Submitted July 31, 1997 (PCT NL9700449) and published February 11, 1999 (WO 99/06440).
- Confirmed favorable immunology, pharmacokinetics and toxicology of BP2 in preparation for an IND application and Phase I clinical trials.
- Examined and confirmed further potential applications of BP2 as an antitumor, antiviral and antiparasitic agent.
- Participated in international scientific conferences and symposia by invitation (Gordon Research Conference, 1999, and Benzon Symposium 1999).
- Maintained a high level of professional expertise through familiarity with current scientific literature and established researchers in the field.
- Coordinated and supervised activities of associate scientists and research technicians

Plasma Lipoproteins and Infection (1997-present)

- Initiated, established and coordinated a project to investigate the mechanism whereby bacterial infection promotes alterations in lipoprotein metabolism which may exacerbate ongoing systemic inflammation.
- Examined the binding selectivity, capacity and kinetics of human plasma lipoproteins for endotoxin *in vitro* with the use of fluorescently labeled LPS and LTA of different chemotypes using High Performance Gel Permeation Chromatography (HPGC).
- Demonstrated efficacy of the lead peptide BP2 in effective competition with lipoproteins for endotoxin sequestration in whole blood.
- Supervised the activities of Ph.D. students and research technicians.

Associate Scientist (1986-1994)

Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam

Mitochondrial biogenesis in Ascomycetes

• Initiated and established a project to investigate the molecular basis for carbon-source-dependent control of cellular respiration in the yeast *Saccharomyces cerevisiae.*

• Molecular cloning, sequence, structural and functional analysis of a number of unique nuclear genes specifying novel components of the mitochondrial translation and respiratory systems.

• Devised and supervised practical teaching programmes in Biochemistry and Molecular Biology for graduate students.

• Coordinated and supervised activities of graduate students and research technicians.

• Participated in specialist meetings and international congresses.

• Engaged in collaborative projects with research laboratories in the U.S.A., Germany, Denmark, Austria and Japan.

Research Associate (1977-1986)

Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam

Nitrogen metabolism and energy generation in enteric bacteria

• Established an investigation into the structural properties and topography of respiratory nitrate reductases from *Escherichia coli* and *Klebsiella aerogenes.*

• Devised and supervised practical teaching programmes in Biochemistry and Molecular Biology for graduate students.

• Coordinated and supervised activities of research technicians.

• Consultant in the use of Apple Macintosh and VAX computers for biological computing.

Medical Biochemist (1975-1977)

Dept. of Chemical Pathology, Red Cross War Memorial Children's Hospital, Cape Town, R.S.A.

Lipoprotein metabolism

• Established a project to investigate the molecular basis for defective plasma lipoprotein lipase in patients with Type I and II hypercholesterolemia.

Education

Ph.D. (1993),
Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam (1988-1992)
Advisors: Prof. H. A. Raué and Prof. R. J. Planta†

M.Sc. (1986),
Dept. of Biochemistry, Vrije Universiteit, Amsterdam (1982-1986)
Major: Biochemistry,
Minors: Analytical Chemistry and Immunology
Advisor: Prof. R. J. Planta†

B.Sc. (Honours)(1974),
Dept. of Biochemistry, University of Cape Town, R.S.A.(1971-1974)
Major: Biochemistry
Minor: Microbiology
Advisor: Prof. C. von Holt†

Awards and honours

• Grant Reviewer, Dutch Heart Foundation, Section: Infection and Atherosclerosis (1999-present).

• Member of the International Endotoxin Society (1998-present).

• Postdoctoral Research Fellowship, Sepsis Fund, Academic Medical Center, Amsterdam (1997-2001).

• Honorary Fellowship of the Dutch Society for Infection & Immunity (1997-present).

• Member of the Dutch Society for Biochemistry and Molecular Biology (1986-present).

Recent invited presentations

Novel Synthetic Peptides with Antimicrobial and Antiendotoxin Properties. Benzon Symposium No. 46, Molecular Mechanisms of Innate Immunity, Copenhagen, Denmark, August 22-26, 1999. *Cited in* Immunology Today. 2000. 2, 68-70.

Therapeutic Potential of Designer Antimicrobial Peptides in Preclinical Models of Infection. 2nd Gordon Research Conference on Antimicrobial Peptides, Il Ciocco, Barga, Italy, April 25-30, 1999.

Novel Antimicrobial Peptides with Antiendotoxin Properties. ALIFI Symposium 'Antimicrobial Peptides', Leiden University Hospital, Leiden, The Netherlands, October 7, 1997.

Recent accompanied presentations and abstracts

Levels, J.H.M., Abraham, P.R., Barreveld, H.P., van den Ende, A.E., van Deventer, S.J.H. *Endotoxin is sequestered by HDL with alpha-mobility.* Satellite Symposium of the XII International Symposium on Atherosclerosis: High Density Lipoproteins and Atherosclerosis, Helsinki, 30 June-3 July 2000.

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Representative recent publications

Abraham, P.R, Appelmelk, B.J and van Deventer, S.J.H. Activity and therapeutic potential of novel antimicrobial peptides with antiendotoxin properties. *J. Biol. Chem.*, submitted.

Levels, J.H.M., Abraham, P.R., van den Ende, A.E. and van Deventer, S.J.H. Distribution and kinetics of lipoprotein associated endotoxin. *Infect. Immun.* 2001, 69, 2821-2828.

Abraham, P.R., Appelmelk, B.J. and van Deventer, S.J.H. Novel synthetic peptides with antimicrobial and endotoxin neutralizing properties for management of the sepsis syndrome. *International patent.* WO 9906440C1, 1999.

Appelmelk, B.J., Qing, A.Y., Helmerhorst, E.J., Maaskant, J.J, Abraham, P.R. Thijs, L.G., McLaren, D.M. Diversity in lipid A binding ligands: comparison of lipid A monoclonal antibodies with $rBPI_{23}$. *Prog. Clin. Biol. Res.* 1995. 392, 453-463.

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Cationic peptides: effectors in innate immunity and novel antimicrobials

Robert E W Hancock

Cationic antimicrobial peptides are produced by all organisms, from plants and insects to human beings, as a major part of their immediately effective, non-specific defences against infections. With the increasing development of antibiotic resistance among key bacterial pathogens, there is an urgent need to discover novel classes of antibiotics. Therefore, cationic peptides are being developed through clinical trials as anti-infective agents. In addition to their ability to kill microbes, these peptides seem to have effector functions in innate immunity and can upregulate the expression of multiple genes in eukaryotic cells. One such function might involve the dampening of signalling by bacterial molecules such as lipopolysaccharide and lipoteichoic acid.
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Since their introduction into human medicine, antibiotics have had an enormous impact on treatment of infectious diseases and the success of invasive medical procedures, such as surgery and chemotherapy. However, the rise in antibiotic resistance threatens to reverse some of these gains.[1] One reason for this development is the paucity of truly novel antibiotics since the introduction of quinolones in the early 1960s. Indeed, for more than 30 years until the release of the streptogramins, synercid, and the oxazolidinone linezolid during the past 18 months, there were no new antibiotic chemical structures. Unfortunately, these antibiotics are niche drugs developed for antibiotic-resistant Gram-positive pathogens, and their restricted activity ranges and toxicity concerns somewhat limit their impact. Thus, it is important to consider new classes of antibiotics. One source is "nature's antibiotics,"[2] the cationic peptides. In this review, I discuss the role of these peptides in innate immunity, and their use as templates in development of a new class of antibiotics that are in phase III clinical trials of topical therapy.

About 20 years ago, the lymph of insects, the granules of human neutrophils, and the skin of frogs were shown to contain peptides that could kill bacteria in culture. Since then, more than 600 cationic peptides have been observed in virtually all species, including bacteria, fungi, insects, tunicates, amphibians, crustaceans, birds, fish, mammals, and human beings.[3] They have generally been referred to as cationic antimicrobial peptides, but in addition to their ability to kill microorganisms directly,[4,5] these substances seem to be able to recruit and promote other elements of host immunity, particularly innate immunity.[6–9] The term cationic amphiphilic peptides, abbreviated to cationic peptides, is therefore used here.

Nature and distribution

Cationic peptides have an enormous variety of sequences and structures,[3] but certain features are common.[2,3,5] The natural cationic peptides are generally 12–50 aminoacids in length, have a net positive charge due an excess of basic lysine and arginine residues over acidic residues, and contain around 50% hydrophobic aminoacids. They fold, owing to the presence of disulphide bridges or contact with membranes, into three-dimensional amphiphilic structures in which the positively charged and hydrophilic domain(s) are well separated from the hydrophobic domain(s). Such a molecule is well suited to interacting with membranes, especially bacterial membranes with their negatively charged and hydrophilic head groups and hydrophobic cores. Nevertheless, both the secondary structures of the cationic peptides, which fit into four classes, and their aminoacid sequences, even within a given class of secondary structures, are quite heterogeneous. The four structural classes include β-sheet molecules stabilised by two or three disulphide bonds, amphipathic α-helices, extended molecules, and loops due to a single disulphide bond (figure 1; the last three classes form upon membrane interaction). The β-sheet and α-helical molecules are by far the most common in nature. Table 1 describes a few representative molecules from nature and related synthetic molecules.

Antimicrobial activities

In the past, with very few exceptions, antibiotics did not have activity against fungi and antifungal drugs did not act against bacteria. However, cationic peptides have a startling range of antimicrobial activities that can include action against most Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites (table 2). Table 1 presents the minimum inhibitory concentrations (MIC) of representative peptides for a Gram-negative bacterium (*Escherichia coli*), a Gram-positive bacterium (*Staphylococcus aureus*), and a fungal pathogen (*Candida albicans*). Various methods are routinely used to assess the ability of peptides to kill bacteria. However, the gold standard is becoming the National Committee for Clinical Laboratory Standards broth dilution method,[11] modified slightly to avoid binding of peptides to plastic surfaces. Generally, the best cationic

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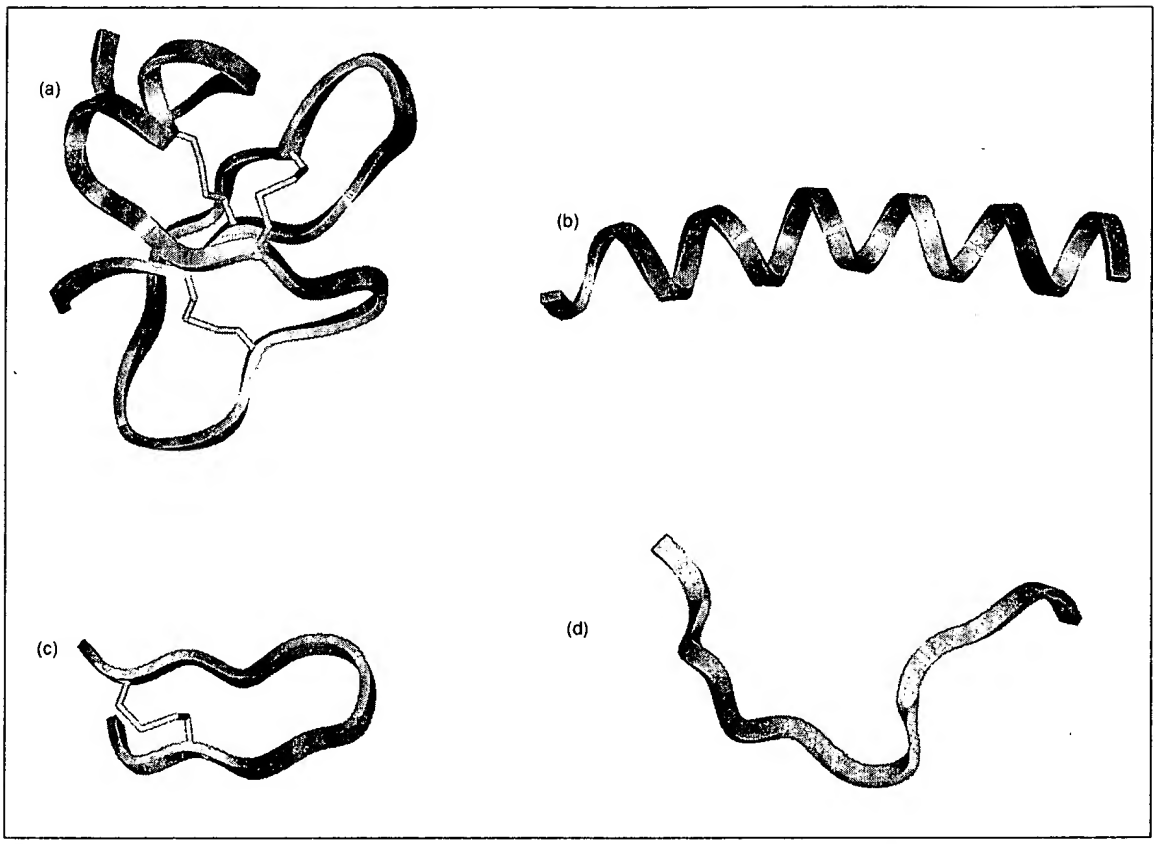

Figure 1. Molecular models of the different structural classes of cationic peptides. These models (taken from the NMR structural database) are based on two-dimensional nuclear magnetic resonance spectroscopy of the peptides in aqueous solution for human β-defensin-2 (HBD-2) or a membrane mimetic condition (other peptides). (a) HBD-2 (PDB code 1FQQ), which forms a triple-stranded β-sheet structure (containing a small α-helical segment at the N-terminus) stabilised by three cysteine disulphide bridges. (b) The amphipathic α-helical structure of magainin 2 (PDB code 2MAG). (c) The β-turn loop structure of bovine bactenecin (model based on the published structure[19]). (d) The extended boat-shaped structure of bovine indolicidin (PDB code 1G89). The backbone structures are shown with the charged regions in blue and the hydrophobic residues in green.

peptides have good activities against most bacteria, and excellent activities (MIC of 1–4 μg/mL) against highly resistant bacteria such as multidrug-resistant *Pseudomonas aeruginosa*, meticillin-resistant *S aureus*, and *Stenotrophomonas maltophilia*.[15,17,18] The cationic peptides are not affected by antibiotic-resistance mechanisms that are limiting the use of other antibiotics; for example, they are as active against meticillin-resistant *S aureus* as they are against meticillin-sensitive strains. A few antibiotic-resistance mechanisms that affect antimicrobial peptides have been described,[19–21] but most seem to have only a moderate (two to four fold) effect on MIC. Indeed, selection of resistant mutants against most peptides is quite difficult, with more than 12 passages on 50% MIC of antibiotic being required to increase resistance by two fold. However, there are a few resistant bacterial species, including *Burkholderia cepacia* (by virtue of its unique outer membrane[14]) and *Serratia* spp.

Cationic peptides are bactericidal, with the MIC and minimum bactericidal concentration coinciding or differing by no more than two fold. They kill bacteria very rapidly (figure 2) compared with conventional bactericidal antibiotics.[15,17]

A large number of, but by no means all, cationic peptides have useful antifungal activities. Indeed, given the importance of fungal diseases of plants, it is perhaps not surprising that many plant peptides are selective for fungi,[5] as are certain insect peptides such as drosomycin.[6] Few studies have been done to study the antifungal spectrum of cationic peptides, and we know little about the specific mechanism of action, although various processes have been described, including morphological distortions, rapid ion fluxes,[23] and inhibition of energised mitochondria.[24]

Another target that has been even more poorly defined is eukaryotic parasites. Selected peptides have activity against protozoa, including trypanosomes, malaria parasites, and nematodes.[25–27] Activity against cancer cells has also been reported,[28,29] although there is doubt as to whether such peptides have the necessary selectivity for malignant over normal cells, and some peptides can be quite toxic. Some peptides, including defensins, indolicidin, polyphemusin, and melittin, also have activity against viruses including HIV, herpes simplex virus, influenza A virus, and vesicular stomatitis virus.[30] Mechanisms have been reported to include blockage of virus-cell fusion and the activity of HIV long terminal repeats.

Table 1. Sequences and properties of selected natural and synthetic cationic peptides

Peptide	Class*	Derivation	Sequence†	MIC (mg/mL)‡ *Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
HNP1	β3(α)	Human neutrophils	AC$_1$YC$_2$**R**IPAC$_3$IAGE**RR**YGTC$_3$IYQGRLWAFC$_2$C$_1$	>50	3·1§	
HBD-3	β3(β)	Human skin	GIINTL**QK**YYC$_1$**R**VRGG**R**C$_2$AVLSC$_3$LP**K**EEQIG**K**C$_2$ ST**R**G**RK**C$_1$C$_3$**RRKK**	~6‖	~12	~6
Polyphemusin I	β2	Horseshoe crab	**RR**WC$_1$F**R**VC$_2$YRGFC$_2$Y**RK**C$_1$**R**-NH$_2$	0·13	0·5	1
Protegrin	β2	Pig	**RGGRL**C$_1$YC$_2$**RRR**FC$_2$VC$_1$VG**R**-NH$_2$	0·5	2	4
IB-367	β2	Synthetic	**R**GGLC$_1$YC$_2$**R**G**R**FC$_1$VC$_2$VG**R**-NH$_2$	1	4	32
Magainin II	α	Frog	GIG**K**FLHSA**KK**FG**K**AFVGEIMNS	50	>100	
MSI-78	α	Synthetic	GIG**K**FL**KK**A**KK**FG**K**AFVKIL**KK**-NH2	2	16	
Cecropin B	α	Silk moth	**K**WKVF**KK**IEK**M**GR**N**IR**N**GIV**K**AGPAIAVLGEA**K**AL-NH$_2$	5	>200	
CP-α2	α	Synthetic	**K**W**KK**FI**KK**IGIGAVL**K**VLTTGLPAL**K**LT**KK**	2	16	64
Indolicidin	E	Bovine neutrophils	ILPW**K**WPWWPW**RR**-NH$_2$	16	8	4
CP-11CN	E	Synthetic	IL**KK**WPWWPW**RRK**-NH$_2$	4	16	16
CP-10A	α	Synthetic	ILAW**K**WAWWAW**RR**-NH$_2$	8	4	16
Bactenecin	C	Bovine neutrophils	**R**LC$_1$**R**IVVIRVC$_1$**R**	8	32	64
BacW2R	C	Synthetic	**RR**LC$_1$**R**IVW**V**I**R**VC$_1$**R**	2	2	>64
Gramicidin S	βC	Bacteria	Cyclic (**LO**VPF^d**LO**VPF^d)	8	2	2
Polymyxin B	CL	Bacteria	Isooctanoyl **BTBB**(BF^d**LBBT**) cyclised	0·5	32	32

*Classes are: β, beta-structured (number refers to the number of disulphide bridges; α or β after the number refers to the family of mammalian α or β defensins from which the peptides come); α, amphipathic α-helical; E, extended structure; C, cyclic; L (polymyxin only), lipopeptide. †One-letter aminoacid code with the following additions. Residues positively charged at neutral pH are in bold. Parentheses indicate aminoacids that are cyclised. Superscript d represents the D-enantiomer; all other aminoacids are L-form. The subscript numbers represent aminoacids that are joined by cysteine disulphides. O, ornithine; B, diaminobutyrate; ×, 2,3-didehydrobutyrine; U, 2,3-didehydroalanine; Z, a-aminobutyrate. ‡MICs were generally determined by modified NCCLS broth dilution assays,[11] and results from the our laboratory are generally used for consistency. Inhibitory concentrations are greatly affected by the method used and the salt content of the assay medium. By the radial diffusion assay method of Lehrer and colleagues, killing at much lower concentrations can be demonstrated. §MICs were not done for *S aureus* but were done for another Gram-positive bacterium *Enterobacter cloacae*,[19] and I assumed here that they are similar. ‖Concentrations resulting in 100% killing.[19]

Peptides are generally found at quite low concentrations in the normal tissues of mammals, and several different peptides can be found in a single tissue.[8] Indeed, their natural role may involve synergy both with each other and with other agents in the host. In frogs, magainin 2 shows synergistic killing with the peptide PGLa,[31] and this finding has been extended by checkerboard titration studies with various cationic peptides.[12] Synergy has also been shown with lysozyme,[12] with various antibiotics against selected wild-type and mutant bacteria,[14] and with antifungal agents, antiprotozoal agents, and the anticancer drug doxorubicin against fungi, protozoa, and cancer cells, respectively.

Mechanism of antibacterial action

An enormous amount of work has been invested in model membrane studies.[32–35] However, although the findings of such studies are consistent with the central observation that the interaction of cationic peptides with the bacterial cytoplasmic membrane is an essential step in the peptides' bactericidal activity, they have also led to a broad variety of hypotheses to explain bacterial killing. Gram-negative bacteria have an additional outer membrane barrier to cross, and the self-promoted uptake hypothesis[36] appears to describe how such uptake occurs (figure 3). According to this hypothesis, the cationic peptides interact with the highly negatively charged surface of the outer membrane and displace magnesium ions that normally partly neutralise this charge. The high negative charge is carried by the anionic glycolipid lipopolysaccharide, which fills the outer monolayer of the outer membrane. The cationic peptide then distorts the outer membrane either by strongly binding to the lipopolysaccharide or by neutralising charge over a patch of the outer membrane. The peptide is then proposed to insert into and translocate across this bilayer. Since many cationic peptides are selective for Gram-negative over

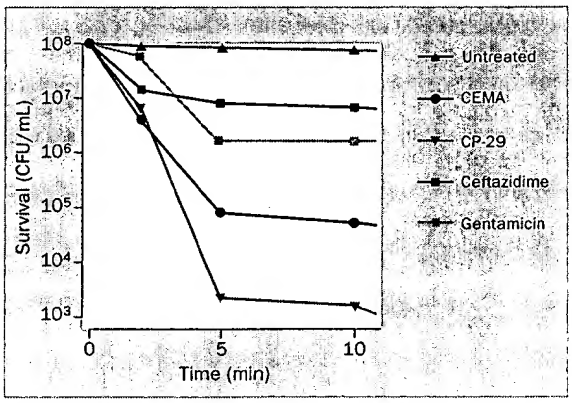

Figure 2. Rate of killing of E coli *UB1005 in Mueller-Hinton broth by four fold the MIC of cationic peptides compared with conventional antibiotics Adapted from published data.[22] Ceftazidime at 2 μg/mL, gentamicin at 0·5 μg/mL, CEMA at 4 μg/mL, and CP-29 at 2 μg/mL.*

Table 2. Activities of cationic antimicrobial peptides and some examples of peptides with those activities

Activities of antimicrobial peptides	Example peptides*
Broad-spectrum antibacterial	Protegrin, IB-367, MSI-78, indolicidin, CEMA, gramicidin S, polyphemusin
Anti Gram-negative bacteria	Polymyxin B
Anti Gram-positive bacteria	HNP1
Synergy with conventional antibiotics	CEMA, magainin II, MSI-78, IB-367
Antifungal	Protegrin, CEMA, indolicidin, gramicidin S, polyphemusin
Synergy with conventional antifungals	Indolicidin
Antiviral (HIV, HSV)	Indolicidin, polyphemusin, protegrin
Anticancer	CEMA, indolicidin
Synergy with conventional anticancer agents	Indolicidin
Antiparasite	Magainin II, indolicidin
Antiendotoxin	CEMA, polyphemusin variants
Wound healing	Magainins, PR39
Chemotactic	HNP-1

*In addition to the peptides described in table 1, CEMA (previously termed CP28 or MBI-28) is an α-helical peptide.[14] Polyphemusin is a β-hairpin peptide from horseshoe crabs, structurally related to protegrin,[15] and PR39 is an extended peptide.[16]

Gram-positive bacteria, and since their action on the outer membrane causes protrusion of the outer membrane or blebs at discrete points on the cell surface (figure 4), we can assume that this interaction with the outer membrane focuses the peptide to attack discrete areas of the cytoplasmic membrane. Also, the distortion of the outer membrane appears to provide a partial explanation for the synergistic activities of antimicrobial peptides described above. However, these outer-membrane interactions do not result in cell death, because peptides that interact well with the outer membrane, but do not kill cells well, have been demonstrated.[37]

Having crossed the outer membrane (or the thick cell wall in the case of Gram-positive bacteria), the peptides approach the cytoplasmic membrane. Model studies have clearly shown, and virtually all researchers agree, that the peptides interact electrostatically with the anionic surface of the bacterial cytoplasmic membrane and this interaction induces insertion of the peptide into a position parallel to the membrane at the interface of the hydrophilic head groups and hydrophobic fatty acyl chains of the membrane phopholipids.[32–35] During insertion, the peptide folds into a membrane-bound structure, if not already folded as a result of disulphide bridging or passage across the outer membrane. After parallel membrane insertion, four outcomes have been proposed on the basis of model membrane studies and to some extent intact cell studies (figure 4). Although several reviewers have suggested lysis as an outcome, there is little evidence for complete dissolution of the majority of bacterial cells at the minimum effective concentration. The left-hand inset of figure 4 shows intact *E coli* treated with 32 times the MIC of the peptide CEMA, without apparent loss of underlying cell shape. There is, however, a striking change in outer-membrane morphology, which adopts a blistered appearance. The right hand panel of figure 4 shows no loss of integrity in *S epidermidis* despite treatment with ten times the MIC of

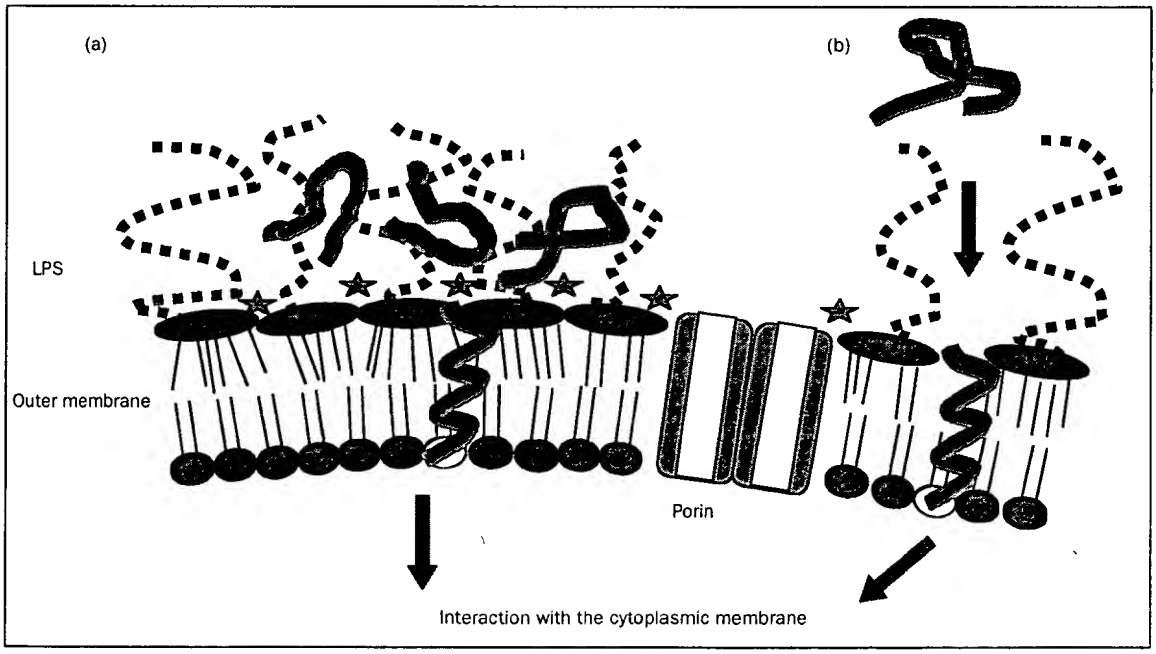

Figure 3. Self-promoted uptake of cationic peptides across the outer membrane. Unfolded cationic peptides are proposed to associate with the negatively charged (mainly due to the presence of highly anionic lipopolysaccharide [LPS]) surface of the outer membrane. They then either neutralise the charge over a patch of outer membrane, creating cracks through which the peptide can cross the outer membrane (a) or actually bind to the divalent cation binding sites on lipopolysaccharide, and disrupt the membrane (b). Once the peptide has crossed the outer membrane, it will interact with the negatively charged surface of the cytoplasmic membrane.

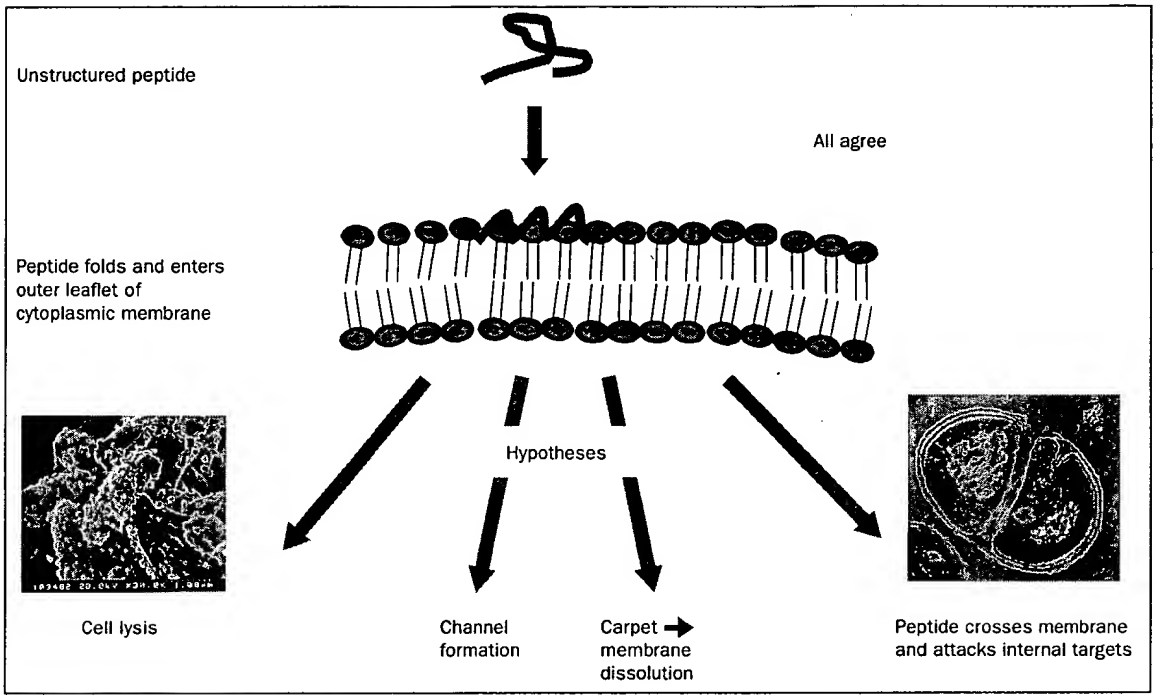

Figure 4. Peptide interaction with the cytoplasmic membrane of bacteria. The mechanism of entry of peptides to cells is undisputed, but there are four hypotheses for how they kill cells. One view is that they lyse cells, but E coli *is not lysed by treatment with 64 µg/mL CEMA for 30 min (inset on left). Another model suggests that when many peptide molecules insert into the membrane interface, they aggregate into a micelle that spans the membrane or flip-flop across the membrane under the influence of the large transmembrane electrical potential gradient;[30,36] inset on right represents the interaction of a linear bactenecin variant Bac2A (with the cysteines changed to alanines) with* Staphylococcus epidermidis. *Some of the events that can be seen are condensation of the DNA indicating uptake of the peptide into the cytoplasm, and cell division defects including an aberrant septum and the initiation of a false septum.*

Bac-2A, and obvious changes to the cell (DNA condensation and a false septum).

Two other hypotheses suggest that the peptides reach a high concentration at the outer surface of the cytoplasmic membrane and reorient to a position perpendicular to the cytoplasmic membrane to form channels with regular structure[33] or cause catastrophic breakdown of cytoplasmic-membrane integrity (the "carpet" model).[35] In these models, the mechanism of action is thought to be breakdown of cytoplasmic-membrane integrity. However, although virtually all cationic amphiphilic peptides cause cytoplasmic-membrane permeabilisation if applied at high enough concentrations, many do not depolarise (break down the membrane potential gradient of) intact cells at concentrations leading to cell killing.[38] Indeed, the toad histone-derived peptide buforin can translocate across lipid bilayers without affecting the membrane barrier function,[39] and all peptides active against Gram-negative organisms are by definition capable of translocation across at least one bilayer, the outer membrane.[32] For this reason, and to explain the results of studies on model membranes and bacterial cytoplasmic-membrane interaction for a wide variety of peptides, we proposed the micellar aggregate channel hypothesis,[32,38] which postulates that the peptides reorient according to concentration and possibly the cytoplasmic-membrane electrical potential gradient (− 140 mV oriented as internal negative) to form micelle-like aggregates that provide informal channels for the movement of ions across the membrane. According to planar bilayer studies,[38] such channels can vary in both size and duration but can last as little time as microseconds; they are proposed to collapse in such a way that the peptide can move to the outer or inner monolayer in a parallel configuration, with the inner monolayer peptide having been translocated. In this model, various targets are possible, including the cytoplasmic-membrane barrier, cell-wall synthesis or degradation, cell division, macromolecular synthesis, or even selective enzyme targets (eg, figure 4, inset on left). Individual peptides might "prefer" a particular target, but

Table 3. Influence of selected cationic antimicrobial peptides given intraperitoneally as a single dose of 8 mg/kg to neutropenic mice infected with *P aeruginosa* and to galactosamine-sensitised mice treated with endotoxin

	Survival (%)	
Peptide*	*P aeruginosa* infection	Endotoxin
None	6	0
CEMA	43	78
CPα2	80	ND
Polyphemusin	20	10
PV5	40	50

*Peptides are the cecropin-melittin hybrid α-helical peptides CEMA and CPa2[14] and horseshoe crab polyphemusin and a variant PV5.[15] ND=not determined.

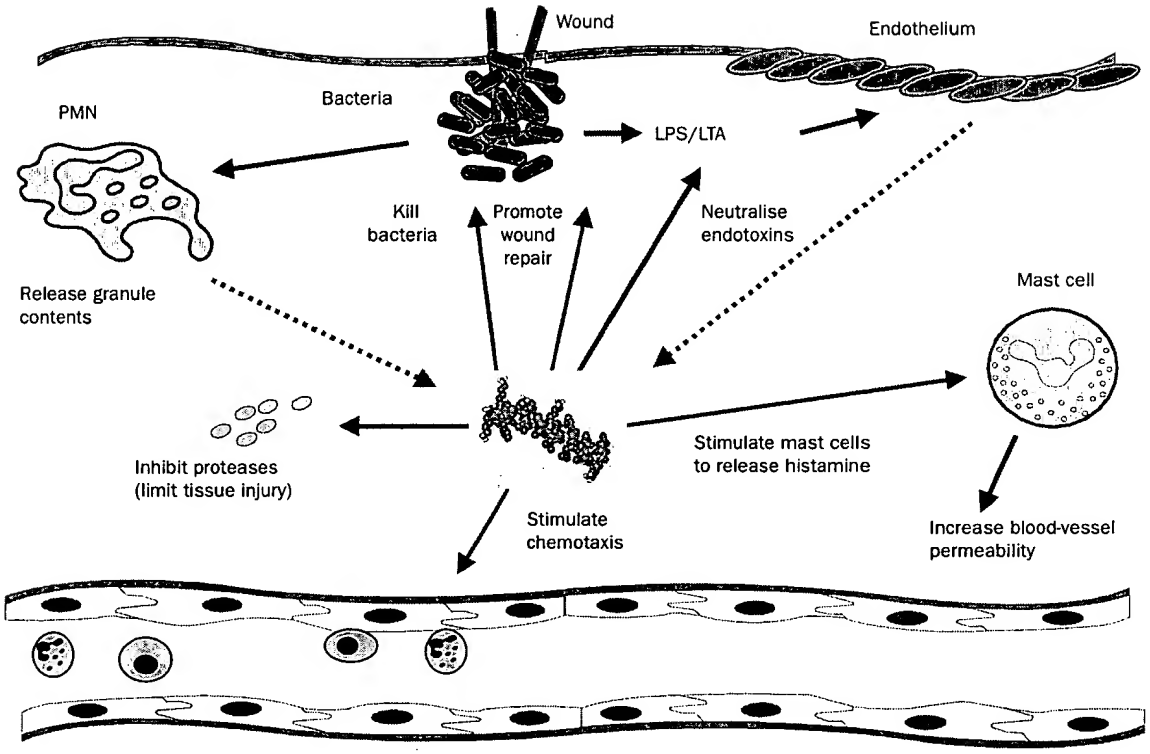

Figure 5. Scheme illustrating the proposed role of cationic peptides in innate immunity with specific reference to events that occur in chronic inflammation. Dotted arrows represent events that lead to increased production of extracellular cationic peptides, solid red lines actions of the peptides, and solid pink lines events due to the bacteria. The overall scheme presented is a mosaic of the separate effects.[7,8] LPS=lipopolysaccharide; LTA= lipoteichoic acid; PMN=polymorphonuclear leucocytes.

the existence of secondary targets and the proposed dependence of these targets on physical interactions (charge–charge and hydrophobic interactions) might help to explain why development of resistance against cationic peptides is difficult. The net effect is that some monomers will be translocated into the cytoplasm[39] and can dissociate from the membrane and bind to cellular polyanions such as nucleic acids.[22]

Role in innate immunity

There is much evidence that cationic peptides have an important role in living hosts.[6-9,40] Since a single host can contain up to about 35 different antimicrobial peptides from all structural classes,[3] elimination of all of these peptides at once is not possible, so clever manipulations must be made to assess in-vivo importance. For example, in drosophila, mutations in regulatory or signalling genes can affect the expression of many peptides and increase susceptibility to bacterial or fungal infections.[41] In mice, Wilson and colleagues[42] identified the enzyme matrilysin, which brings about processing of intestinal preprodefensins to mature defensins. Knocking out the matrilysin gene denuded the small intestine of mature defensins and increased susceptibility to infection with ingested organisms by ten fold. Cole and colleagues[43] similarly applied protease inhibitors specific for the protegrin-processing enzyme to wounds on the skin of pigs, decreasing the amounts of active protegrin and the ability to resist a bacterial challenge. Although similar experiments cannot be done in healthy human beings, patients with specific granule deficiency syndrome lack α-defensins and have severe and frequent bacterial infections.[5] Another way of assessing activity in vivo is to increase the amount of cationic peptides. This has been done in two ways. Bals and co-workers[44] introduced an adenovirus carrying the transgene for the human peptide LL-37 into the lungs of mice, leading to protection against *E coli* infections and endotoxin. Many other studies have shown that exogenously introduced peptides can protect against endotoxaemia and bacterial and fungal infections (table 3).[6,9]

There is no doubt that cationic peptides can be found in high (bactericidal) concentrations at certain sites in the host.[8,9] For example, the concentration of defensins in the azurophilic granules of neutrophils can be as high as 10 mg/mL, whereas various insect peptides when induced can circulate in the lymph at concentrations of up to 100 μg/mL or more. In these cases, we can assume that such peptides function in innate immunity to kill infectious agents directly. On the other hand, certain body sites in human beings contain quite low concentrations of peptides (eg, airway surface fluids contain 0·3 to 8 μg/mL of human β-defensin-2[45,46] and 2 μg/mL of the α-helical cathelicidin LL-37[47]). These concentrations can be increased for some peptides by infection, but except in pathological,

inflammatory diseases, concentrations still seem to be below those needed to kill infectious agents. Thus, the fact that these peptides have various activities that are relevant to innate immunity is of great interest.

In particular, peptides can neutralise host responses to conserved bacterial signalling molecules such as endotoxic lipopolysaccharide from Gram-negative bacteria,[48] lipoteichoic acid from Gram-positive bacteria,[9] and unmethylated CpG DNA from all bacteria (MG Scott, REW Hancock, unpublished). Such molecules interact with Toll-like receptors on the surface of host cells to trigger signalling cascades and cause upregulation of cytokines, such as tumour necrosis factor (TNF) and interleukin 6, chemokines like macrophage inflammatory protein 1α and 1β, and dozens of other gene products.[48] Although low concentrations of these signalling molecules cause beneficial proinflammatory responses and fever, too sustained or vigorous a response can lead to systemic circulation problems, organ failure, and even death.[49] Cationic peptides can neutralise these responses, for example by suppressing the upregulation by lipopolysaccharide of TNF expression both in macrophages in culture and in sensitised mice.[14] This action results in protection against endotoxaemia and death.[50] The mechanism of suppression involves both inhibition of binding of lipopolysaccharide to a serum factor lipopolysaccharide-binding protein, in addition to a proposed direct action on host cells.[50] Microarray experiments showed that the effects of the cationic peptides CEMA[48] and LL-37[50] are selective, in that of the 52 genes observed to be upregulated by lipopolysaccharide, only about 35 were suppressed to differing extents in the presence of cationic peptides. Since the natural bacterial flora of animals can conceivably release small amounts of bacterial signalling molecules, one role of cationic peptides at the surface of cells may thus be to prevent the induction of inflammatory responses by these bacteria.

Cationic peptides also have various interactions that relate to innate immunity, including stimulation of the chemoattraction of monocytes and neutrophils, promotion of histamine release from mast cells, inhibition of tissue proteases, and stimulation of wound healing (figure 5). Microarray experiments have confirmed the ability of cationic peptides to upregulate selectively the expression of more than 30 genes.[48] Furthermore, there is strong circumstantial evidence for involvement of specific receptors in the chemotactic response stimulated by peptides.[47] Nevertheless, to date these results remain fragmentary and there are as yet no data in animal models to confirm that such interactions are important.

Clinical development

The general proof of principle for the use of cationic antimicrobial peptides as therapeutic agents has already been established.[2] Two bacterium-derived, non-ribosomally synthesised cationic peptides, gramicidin S and polymyxin B,[52] have already found use in topical creams and solutions. However, these molecules tend to be toxic and this characteristic limits their potential for systemic use. Interestingly, neutralisation of the amino groups of polymyxin E with methane sulphonate creates a prodrug, colomycin, that can be used systemically.

Cationic peptides have had a chequered history in the clinic and currently only five clinical trials of topical treatment are underway. These include a phase III trial for therapy, by a protegrin-like molecule, IB-367, of oral mucositis, a painful ulcerative polymicrobial infection most commonly associated with radiotherapy or chemotherapy for cancer. There were also phase II clinical trials of IB-367 in aerosol formulation for *P aeruginosa* lung infections in people with cystic fibrosis. An indolicidin, MBI-226, is undergoing phase III clinical trials for sterilisation of insertion sites for central venous catheters; these trials have been fast-tracked by the US Food and Drug Admistration. Other indolicidin-like peptides are being investigated for therapy of acute acne (in phase II clinical trials).

However, until cationic amphiphilic peptides can be used systemically they will not achieve their true potential, and the barriers that must be overcome are discussed below.

Barriers

Any new class of pharmaceuticals faces a series of tests that must be overcome to achieve success in the clinic. In general, these include demonstration of good activity, appropriate formulation, an appropriate manufacturing method, sufficient stability in vivo, and low toxicity. Since there are virtually no published data on many of these topics for cationic peptides, the following discussion is somewhat speculative.

There is no doubt that cationic amphiphilic peptides have excellent antimicrobial activity in vitro and, in principle, represent almost ideal candidate drugs. There is some evidence that this good in-vitro activity can translate to in-vivo activity in animals, but studies in which protection is complete are rare, probably owing to formulation or stability issues. An example is observed for polyphemusin I, a β-hairpin peptide from horseshoe crabs, which in vitro is the most active peptide we have studied to date, but in animal models has no activity against infections.[15] By contrast, modest sequence modifications can create peptides with slightly lower in-vitro activity but reasonable (although incomplete) protection in infections of animals.[15] The most obvious cause of poor or incomplete in-vivo activity is lack of stability due to the action of host proteases. Ways of overcoming this instability might be improved formulation (eg, in liposomes, masking the peptide), use of the prodrug approach as discussed above for colomycin, development of cyclic peptides with strained peptide bonds that are more resistant to proteolysis, and sequence modifications. In the last case,[51] cationic peptide precursors can be protease inhibitors,[52] so moderate changes in sequence might convert a protease substrate to a protease inhibitor.

Search strategy and selection criteria

I have followed the field of antimicrobial peptides closely for the past 15 years with an automated monthly search of *Current Contents* and *Biological Abstracts*, using key words that include general terms such as antimicrobial peptides and the names of specific peptides of note. In addition, manual searches of Medline were carried out, and the press releases of the major companies involved in the field were examined. The amount of literature in this field is becoming voluminous, so the references used were highly selected to present specific points of note, and many general review articles rather than the first paper presenting a given topic. I recommend that readers also examine review articles referenced herein to find many other excellent papers.

Conclusion

Antimicrobial peptides offer an enormous range of useful activities ranging from antimicrobial to immunomodulation. They are proceeding to the clinic as topical antibiotic agents. However, elucidation of their biological importance in innate immunity and realisation of their full clinical potential will require much more effort.

Acknowledgments

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52 Varbanac D, Zanetti M, Romeo D. Chemotactic and protease-inhibiting activities of antibiotic peptide precursors. *FEBS Lett* 1993; 317: 255–58.

Curriculum vitae

Philip Richard Abraham

January 3, 1950, Cape Town

In de Papiermolen 30
1115 GS Duivendrecht,
The Netherlands

Appointments and Experience

Senior Scientist (1995-present)
Dept. of Experimental Internal Medicine , Academic Medical Center, University of Amsterdam,
Postdoctoral Scientist (1994-1995)
Dept. of Medical Microbiology, Vrije Universiteit, Amsterdam

Anti-infective Peptide Therapeutics (1994-present)

- Initiated and established a project for the development of novel synthetic antimicrobial peptide therapeutics for the prevention/treatment of septic shock caused by multi-drug resistant bacterial pathogens.
- Generated a new class of Bactericidal Peptides (BP), with antibiotic and anti-inflammatory activity by rational design and molecular modeling.
- Demonstrated potent broad-spectrum bactericidal activity against multi-resistant clinical isolates, high-affinity lipopolysaccharide (LPS) and lipoteichoic acid (LTA) -binding and effective endotoxin-neutralizing activity of a number of candidate peptides in preclinical studies.
- Demonstrated potential therapeutic properties of the lead peptide BP2 in animal models of experimental Gram-negative endotoxemia, peritonitis, pneumonia and Gram-positive bacteremia.
- Prepared an international patent application "Novel synthetic peptides with antimicrobial and endotoxin-neutralizing properties for management of the sepsis syndrome". Submitted July 31, 1997 (PCT NL9700449) and published February 11, 1999 (WO 99/06440).
- Confirmed favorable immunology, pharmacokinetics and toxicology of BP2 in preparation for an IND application and Phase I clinical trials.
- Examined and confirmed further potential applications of BP2 as an antitumor, antiviral and antiparasitic agent.
- Participated in international scientific conferences and symposia by invitation (Gordon Research Conference, 1999, and Benzon Symposium 1999).
- Maintained a high level of professional expertise through familiarity with current scientific literature and established researchers in the field.
- Coordinated and supervised activities of associate scientists and research technicians

Plasma Lipoproteins and Infection (1997-present)

- Initiated, established and coordinated a project to investigate the mechanism whereby bacterial infection promotes alterations in lipoprotein metabolism which may exacerbate ongoing systemic inflammation.
- Examined the binding selectivity, capacity and kinetics of human plasma lipoproteins for endotoxin *in vitro* with the use of fluorescently labeled LPS and LTA of different chemotypes using High Performance Gel Permeation Chromatography (HPGC).
- Demonstrated efficacy of the lead peptide BP2 in effective competition with lipoproteins for endotoxin sequestration in whole blood.
- Supervised the activities of Ph.D. students and research technicians.

Associate Scientist (1986-1994)

Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam

Mitochondrial biogenesis in Ascomycetes

• Initiated and established a project to investigate the molecular basis for carbon-source-dependent control of cellular respiration in the yeast *Saccharomyces cerevisiae.*

• Molecular cloning, sequence, structural and functional analysis of a number of unique nuclear genes specifying novel components of the mitochondrial translation and respiratory systems.

• Devised and supervised practical teaching programmes in Biochemistry and Molecular Biology for graduate students.

• Coordinated and supervised activities of graduate students and research technicians.

• Participated in specialist meetings and international congresses.

• Engaged in collaborative projects with research laboratories in the U.S.A., Germany, Denmark, Austria and Japan.

Research Associate (1977-1986)

Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam

Nitrogen metabolism and energy generation in enteric bacteria

• Established an investigation into the structural properties and topography of respiratory nitrate reductases from *Escherichia coli* and *Klebsiella aerogenes.*

• Devised and supervised practical teaching programmes in Biochemistry and Molecular Biology for graduate students.

• Coordinated and supervised activities of research technicians.

• Consultant in the use of Apple Macintosh and VAX computers for biological computing.

Medical Biochemist (1975-1977)

Dept. of Chemical Pathology, Red Cross War Memorial Children's Hospital, Cape Town, R.S.A.

Lipoprotein metabolism

• Established a project to investigate the molecular basis for defective plasma lipoprotein lipase in patients with Type I and II hypercholesterolemia.

Education

Ph.D. (1993),
Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam (1988-1992)
Advisors: Prof. H. A. Raué and Prof. R. J. Planta†

M.Sc. (1986),
Dept. of Biochemistry, Vrije Universiteit, Amsterdam (1982-1986)
Major: Biochemistry,
Minors: Analytical Chemistry and Immunology
Advisor: Prof. R. J. Planta†

B.Sc. (Honours)(1974),
Dept. of Biochemistry, University of Cape Town, R.S.A.(1971-1974)
Major: Biochemistry
Minor: Microbiology
Advisor: Prof. C. von Holt†

Awards and honours

• Grant Reviewer, Dutch Heart Foundation, Section: Infection and Atherosclerosis (1999-present).

• Member of the International Endotoxin Society (1998-present).

• Postdoctoral Research Fellowship, Sepsis Fund, Academic Medical Center, Amsterdam (1997-2001).

• Honorary Fellowship of the Dutch Society for Infection & Immunity (1997-present).

• Member of the Dutch Society for Biochemistry and Molecular Biology (1986-present).

Recent invited presentations

Novel Synthetic Peptides with Antimicrobial and Antiendotoxin Properties. Benzon Symposium No. 46, Molecular Mechanisms of Innate Immunity, Copenhagen, Denmark, August 22-26, 1999. *Cited in* Immunology Today. 2000. 2, 68-70.

Therapeutic Potential of Designer Antimicrobial Peptides in Preclinical Models of Infection. 2nd Gordon Research Conference on Antimicrobial Peptides, Il Ciocco, Barga, Italy, April 25-30, 1999.

Novel Antimicrobial Peptides with Antiendotoxin Properties. ALIFI Symposium 'Antimicrobial Peptides', Leiden University Hospital, Leiden, The Netherlands, October 7, 1997.

Recent accompanied presentations and abstracts

Levels, J.H.M., Abraham, P.R., Barreveld, H.P., van den Ende, A.E., van Deventer, S.J.H. *Endotoxin is sequestered by HDL with alpha-mobility.* Satellite Symposium of the XII International Symposium on Atherosclerosis: High Density Lipoproteins and Atherosclerosis, Helsinki, 30 June-3 July 2000.

Abraham, P.R., Appelmelk, B.J. and van Deventer, S.J.H. *Novel synthetic peptides with antimicrobial and antiendotoxin properties.* Benzon Symposium No. 46. Molecular Mechanisms of Innate Immunity. Copenhagen, Denmark, August 22-26, 1999.

Doerffler-Melly, J., Franco, R.F., Groot, A.P., Abraham, P.R., van der Poll, T., ten Cate, H., Reitsma, P.H., and Spek, C.A. *Thrombomodulin deficiency results in enhanced host defense and increased survival in mice.* 41st American Society of Hematology Meeting, New Orleans, December 3-7, 1999, Abstract #2618.

Abraham, P.R., Appelmelk, B.J. and van Deventer, S.J.H. *Novel synthetic peptides with antimicrobial and antiendotoxin properties.* 13th International Endotoxin Conference, Santa Fe, NM, September 12-15, 1998. pg. 111.

Levels, J.H.M., van den Ende, A.E., van Deventer, S.J.H and Abraham, P.R., *Distribution and kinetics of lipoprotein associated endotoxin.* 13th International Endotoxin Conference, Santa Fe, NM, September 12-15, 1998. pg. 119.

Levels, J.H.M., Abraham, P.R., van den Ende, A.E. and van Deventer, S.J.H. *High performance gel permeation chromatography (HPGC) in the analysis of plasma lipoprotein-associated endotoxin.* 50th Annual Meeting of the AACC, Chicago, Ill., August 4-6, 1998, *Clin. Chem.* 44 suppl. 1998, pg. A81.

Representative recent publications

Abraham, P.R, Appelmelk, B.J and van Deventer, S.J.H. Activity and therapeutic potential of novel antimicrobial peptides with antiendotoxin properties. *J. Biol. Chem.*, submitted.

Levels, J.H.M., Abraham, P.R., van den Ende, A.E. and van Deventer, S.J.H. Distribution and kinetics of lipoprotein associated endotoxin. *Infect. Immun.* 2001, 69, 2821-2828.

Abraham, P.R., Appelmelk, B.J. and van Deventer, S.J.H. Novel synthetic peptides with antimicrobial and endotoxin neutralizing properties for management of the sepsis syndrome. *International patent.* WO 9906440C1, 1999.

Appelmelk, B.J., Qing, A.Y., Helmerhorst, E.J., Maaskant, J.J, Abraham, P.R. Thijs, L.G., McLaren, D.M. Diversity in lipid A binding ligands: comparison of lipid A monoclonal antibodies with $rBPI_{23}$. *Prog. Clin. Biol. Res.* 1995. 392, 453-463.

Abraham, P.R., Mulder, A., van't Riet, J. and Raue, H.A. Characterization of the *Saccharomyces cerevisiae* nuclear gene CYB3 encoding a cytochrome *b* polypeptide of respiratory complex II. *Mol. Gen. Genet.* 1994. 242, 708-716.

Abraham, P.R. Molecular characterization of a chromosomal region specifying mitochondrial components in the yeast *Saccharomyces cerevisiae*. Ph.D. Thesis. 1993.

Abraham, P.R., Mulder, A., van't Riet, J., Planta, R.J. and Raue, H.A. Molecular cloning and physical analysis of an 8.2 kb segment of chromosome XI of *Saccharomyces cerevisiae* reveals five tightly linked genes. 1992. *Yeast* 8, 227-238.

Abraham, P.R., Wientjes, F.B., Nanninga, N. and van't Riet, J. Part of nitrate reductase of *Klebsiella aerogenes* is intimately associated with the peptidoglycan. *J. Bact.* 1987. 169:849-855.

References

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